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1978 USAMRIID PLANNING SESSION WITH THE AD HOC STUDY GROUP FOR --ETC(U)
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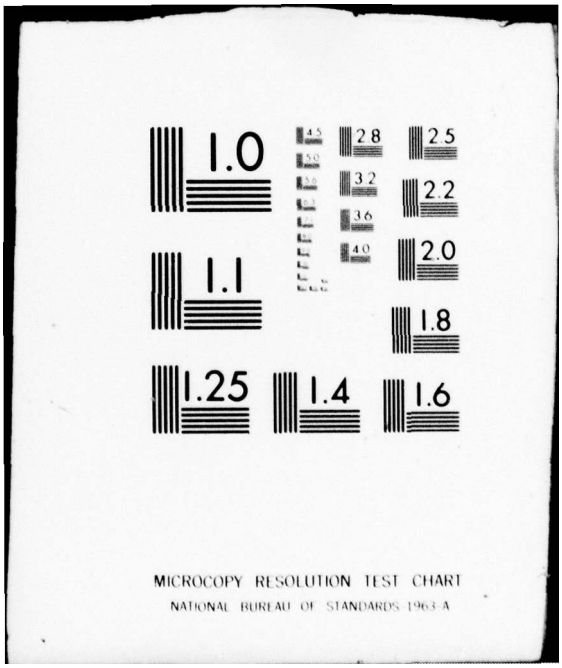
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# 1978 USAMRIID PLANNING SESSION WITH THE AD HOC STUDY GROUP FOR SPECIAL INFECTIOUS DISEASE PROBLEMS

(10) William S. /Augerson, Richard F. /Barquist, Clarence J. /Peters, Robert M. /Rice Francis E. /Cole, JR

7-8 December 1978.

(9) Annual meeting

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USA Medical Research Institute
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Fort Detrick
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# 1978 USAMRIID PLANNING SESSION

# WITH THE

# AD HOC STUDY GROUP

# FOR

# SPECIAL INFECTIOUS DISEASE PROBLEMS

7-8 December 1978

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other official documents.

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FORT DETRICK

FREDERICK, MARYLAND 21701

June 1979

## REPORT DOCUMENTATION PAGE

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| Electron microscopy | Korean hemorrhagic fever | Immunology | Vaccines |
| Lassa fever virus | Francisella tularensis | Botulism | |

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Research Institute of Infectious Diseases

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## Consultants and Guests

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|---|---|
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| Center for Disease Control | D. R. Hopkins, M.D. |
| Aberdeen Proving Ground | Ira Abelow |

# REMARKS BY THE COMMANDER, MRDC

William S. Augerson, MC
Brigadier General, Commanding

My appearance here is partly ceremonial and partly substantive. I would like to say "Welcome." Despite the stories you may have heard, we remain very much in need of your advice and consultative capabilities. Although the trademark on the umbrella under which you have operated may soon be different than before, we hope very much to continue to enjoy your support and attention. I remain not only personally grateful to you, but admire the dedication and sense of humor or irony you have experienced in helping the likes of me and the Army for a very long time. You will have to excuse us, if now and then, we bring up something you thought had been dealt with a decade ago. It is nice to confirm independently these things from time to time. I hope you have a feeling, as I do, that not only is there a lot of science happening here but that, it also has a vector associated with it. These happenings are going somewhere and I look forward to listening as well as talking. I will not bore you with things you already know. The Republic has some serious external as well as internal challenges things to be genuinely concerned about. The annual identification of new and unpleasant disease continues to be a growth industry. Paranoia in the media about any military-biological research remains a good, steady cottage industry and keeps a lot of people in business while it sells magazines. I did not realize that science needed sensationalism, but apparently it does. At the same time, some of our old opponents keep coming back; perhaps in different forms. For example, malaria is still with us and moving around the world. I suppose you noticed the appearance of R-1 resistant malaria in Africa recently. I do not think we can have enormous confidence even with those diseases we think have been competently dealt with. They will not necessarily stay that way. So, I want you to know how greatly I appreciate your forebearance with the ever-changing force of consulting bureaucracy and new federal initiatives. I am sure the forms you fill in, reference the extracurricular activities of your relatives to the fourth generation and they are probably different this year from last. I want to emphasize my appreciation for all of you remaining with this enterprise.

# CURRENT STATUS OF USAMRIID

Richard F. Barquist, M.D.
Commanding Officer

I wish to talk about 3 things this morning: (1) the health of the Institute; (2) a series of news items, significant events of the past year which I would like to summarize; and (3) outline of a tentative concept for BW medical defense, designed to be presented to nonmedical decision-makers of the Army.

At the beginning I wish to reemphasize the policy of the United States, which has no intention to use offensive BW, but to retain a defensive program. "The U.S. shall renounce the use of lethal biological agents and weapons, and all other methods of biological warfare." and "The U.S. will confine its biological research to defensive measures such as immunization and safety measures." (National Policy, 25 Nov 69). This policy has been codified into international law through the 1972 U.N. Biological Weapons Convention with over 100 nations signing. Signatories agree never to acquire biological agents or toxins which have no justification for prophylactic, protective or other peaceful purposes. (U.S. signed, 10 Apr 1972 and ratified 22 Jan 1975). A few nations have not signed: People's Republic of China, Israel, Libya, Cambodia and France. France has passed an internal law which has the same provisions as the convention. A few other nations have signed with certain reservations or have not ratified; Japan and some of the Arab countries have not ratified; the Arabs have said that they would not be bound by the convention if they were at war with Israel. A few other countries have said the convention would not apply if some other country attacked them with BW weapons.

To complete your orientation, I wish to review our mission statement and its emphases on naturally occurring infectious disease as well as on disease of BW potential. The disease agents our program addresses qualify under both aspects.

> "Perform studies on the pathogenesis, diagnosis, prophylaxis, treatment, and epidemiology of naturally occurring infectious diseases of military importance with emphasis on problems associated with the medical defense against biological agents and on those organisms which require special containment facilities."

I would now like to shift to health of the Institute. I will not talk about the research program, because that is what we are going to address ourselves to in great detail. Table I summarizes our funding for the last 2 years, and if I receive no bad news from LTC Pedersen, the news for next year is generally good. For FY 78, we had enough money to do the important things and we spent it all. The growth in funds between 1978 and 1979 is enough to cover inflation and should be adequate. The contract monies, nearly $3,000,000 in FY 78, are also adequate. With this money we buy the talents of some 15 university investigators with skills and capabilities we do not have in-house to amplify our programs. It also buys us the biologics production capabilities of the facility at Swiftwater, PA, which I will discuss. Certainly FY 80 looks good, if it holds; however, there are many ways to lose between now and the appropriation of the money.

TABLE I. USAMRIID FUNDING TREND (20 May 1978)

| | $ x 1,000 | | | |
|---|---|---|---|---|
| | FY 77 | FY 78 | FY 79 | FY 80 |
| In-house | 6,959 | 8,165 | 8,642 | 9,737 |
| Contract | 1,678 | 2,600 | 2,988 | 3,000 |
| Total | 8,637 | 10,765 | 11,630 | 12,737 |
| % In-house | 81 | 76 | 75 | 76 |

Fig. 1 (left) shows how our money was allocated in FY 78 by major program components. About half of the funds went to "Improved Prevention," which is essentially vaccine development. About 1/4 went for "Pathogenesis" and an even smaller share for "Improved Therapy" and "Early Diagnosis." Fig. 1 (right) does the same for the FY 79 plan, and shows a greater amount of money and effort devoted to "Early Diagnosis." "Improved Prevention" remains about the same, "Pathogenesis" a little less, and "Improved Therapy" about the same. Since the program has grown, the absolute dollars have not been reduced as much as the percentage breakdown would indicate.

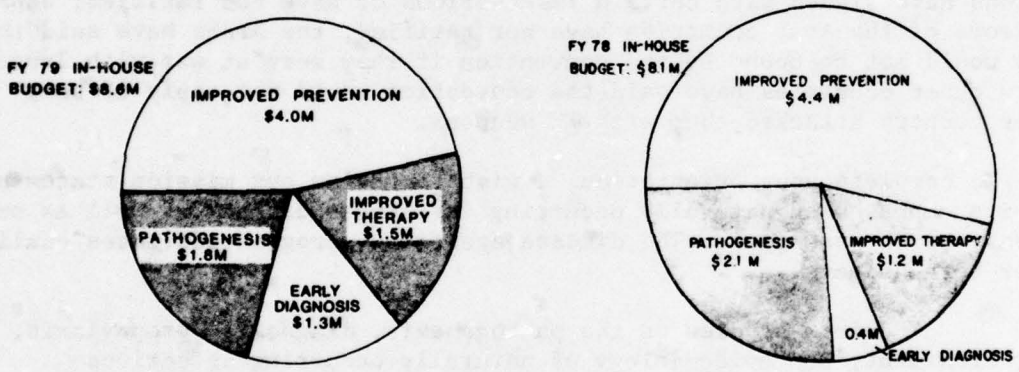


Fig. 1. Distribution of in-house budgets for FY 78 and FY 79

Facilities. Our facilities are superb, although the world is not perfect and things do not always work the way we think they should. I mentioned last year that we were in the process of upgrading many of our internal biohazard containment facilities. This program has not gone as fast as our money should have allowed it to go. We were limited by engineering design talent and time, but it has been moving. In building 1412, we have been able to turn off the incinerator with its high fuel cost. It has been replaced by high-efficiency filters in the exhaust air stream to contain biohazards. The Machupo suite in building 1425 is in the midst of upgrading. This suite, with its cabinets and suit area, is available for inspection. A major accomplishment has been the establishment of a hot, clinical laboratory, which functions, with pressurized suits, in an improvised mode. The hot clinical laboratory is in suite AA5. We also have Lassa fever (LAS) research going on in the same area, so it is not

exactly ideal. Our plan, as soon as the construction is funded, is to upgrade the space in the clinical laboratory, which was designed as 2 parallel suites of rooms, one cold and one hot. What we lack in this area is a suit-shower facility. Clinical laboratory work on hazardous specimens was accelerated by the arrival of 2 LAS suspect patients earlier in the year. Another upgrading we would like to achieve is to convert our isolation suite for patient care, i.e., the 2-patient facility, to full plastic-suited operation. Now, we use only a clean air hood for the attendants, as opposed to fully pressurized suits. We are also establishing a coagulation laboratory under the stimulus of a new hematologist who has just joined us, reinforcing the efforts of Dr. de Sa Pereira.

We will acquire an upgraded automatic controller, a computerized system that monitors the building and will extend that automatic control to building 1412. We will have improved monitoring of many functions in both buildings when this is achieved. This project is well along. We will go through a number of laboratory areas to upgrade filters and enhance air flow in hoods to meet modern standards for both biohazard and chemical hazard containment.

Last of all, we still have pending before Congress, a major modernization of building 1412, which will remove 2 large and now useless aerosol tanks and replace them with good animal holding facilities. This will have the effect of freeing space in the building now dedicated to holding and conditioning our animals. The end-result will provide adequate animal holding plus additional cold laboratory space. I guess I have not seen a project with lower certainty; we have a chance to get it approved, if it ever gets to Congress in FY 80. If this measure does not proceed, we have fall-back concepts, not very viable ones, but they would include trailers and temporary buildings. They are not cheap and not very desirable, but nothing is cheap today. The project would cost the taxpayers $1,400,000, if it is approved.

Equipment. I was surprised when I got the report that we spent over $1,000,000 for equipment in the last year. However, it was money well spent for general upgrading of equipment plus a few big items. For example, we bought a third electron microscope, a Joel (which has not been delivered), with scanning and transmission capabilities. This will allow one of our less efficient scopes to revert to training support for pathology trainees.

Last year, I reported to you that our radiation capability was lost when our 1 million volt x-ray tube was declared defunct and unrepairable. We now have on order 2 small, self-contained gamma radiation sources from Nuclear Canada. One is of a size to allow radiation of cell cultures and small material. The other is a little larger and allows small animal radiation at acute rates of delivery. So the research on the interaction of infectious disease and radiation will continue here.

We have now acquired one each of Trexler-type isolator units manufactured by Vickers Medical of the United Kingdom.

We are in the process of procuring a minicomputer for laboratory application. This will allow real-time recording and analysis of the physiological studies done by Dr. Liu and his colleagues. We see this as a leading edge which will eventually include pathology and other minicomputer applications throughout the Institute.

In order to keep ourselves in good standing with the American Association for Accreditation of Laboratory Animal Care (AAALAC), we have agreed to upgrade cages, and have already procured $221,000 worth of new stainless steel cages which meet these new laboratory standards.

Personnel. The Secretary of the Army and just about everyone else has said the Army is on hard times for physicians. Our recruitment efforts have managed to keep us even with our losses. We are 2 physicians ahead of last year (Table II). The recruitment effort is still active in producing new candidates. We have identified one individual who has agreed to join us in mid-summer. We have several others that are still considering us. Our other scientists, veterinarians, and medical service corps Ph.D. numbers are nearly full strength.

TABLE II. DISTRIBUTION OF PHYSICIANS (1 Dec 1978)

| | Authorized/Assigned | | |
|---|---|---|---|
| | Military | Civilian | Total |
| Research | 14/11[a] | 3/3 | 17/14 |
| Clinic | 1/ 1 | 1/1 | 2/ 2 |
| Total | 15/12 | 4/4 | 19/16 |

[a]One is on permanent loan from WRAIR

For the enlisted laboratory technicians who are a critical resource, we have 95 authorized; we have had no difficulty in replacing Medical Research Volunteer Subjects (Table III). They are volunteering at a rate of about 60-70%, that is, during a 3-year tour, 60-70% will have participated in a research study. I think this testifies to the fact that, indeed, it is a volunteer program and they are not under pressure.

TABLE III. ALL PERSONNEL (1 Oct 78)

| | Authorized | Assigned |
|---|---|---|
| Officers | 74 | 68 |
| Cadre | 139 | 126 |
| Medical Research Volunteer Subjects | 95 | 92 |
| Civilians | 197 | 194 |
| Total | 505 | 480 |

In terms of key personnel, there have been no new division chiefs, in the past year. Colonel German, our Deputy Commander and Special Projects Officer for Early Diagnosis and Laboratory Quality Control, has departed and is now at the Armed Forces Institute of Pathology. LTC Ascher is now at the University of California, Irvine. We have a number of new investigators: 5 are physicians, 9 are MSC officers, 9, veterinarians, and 2, civilians. They are listed by name and division in the handout. I hope you will have a chance to meet them during the next 2 days.

The next topic is the legal status of you, the advisors; this gathering of individual consultants complies with the letter, but certainly not with the spirit of Public Law 92463, the Federal Advisory Act. There is a plan for January 1979, to recharter the committee which advises General Augerson; this rechartering will provide for 40 regular members and 25 associate members, and authorize subcommittees. It is visualized that this gathering will become a subcommittee.

The charter will include the following provisions: limitation to two 2-year terms for regular members or a combination of one 2-year term as an associate and one 2-year term as a regular member; after that, the individual must be out of office for a period of 2 years before he is again eligible for reappointment. At the next meeting we will see: (a) an advance announcement of the meeting to the general public in the Federal Register and (b) a reporting of the minutes to the Library of Congress. There will be other compliance with the Act. Your present status is that we are exploiting a loop-hole which allows ad hoc groups to meet under the provisions of the existing charter of the Medical Research and Development Committee at this time. But you will shift from an ad hoc committee to a subcommittee next year.

Colonel Russell has been named by MRDC as the manager of Mission Area One, disease hazards. Essentially all of the Army work on infectious disease will be the management responsibility of Colonel Russell, as a staff officer for General Augerson. The major components he will supervise will be the USAMRIID program in its entirety and the infectious disease program at WRAIR. As I understand the concept, he will be responsible for big-picture planning. Such things as prioritization of major components of the program and resource allocation, particularly looking ahead. Keep in mind the response time of a major program change is about 2 years. He will look at the balance between the in-house and contract programs, and perhaps at lots of other things dictated by his interests.

It is the intention of MRDC that the Institute and WRAIR merge. The merger has already progressed through step 1. This Institute is already assigned to WRAIR; I report to Colonel Rapmund. Following steps are to coalesce a critical and viable mass of rickettsiology at WRAIR. For a time, it was planned to be consolidated here, but key civilian staff did not want to leave the Washington area. The plan was reworked and rickettsial research will be done at WRAIR, except for Q fever retained here. Another area of coalescence deals with arbovirology. Plans are not far enough along for details to be available; however the concept is to move arbovirus research and supporting entomology here.

There will be changes in the command and administrative functions looking for economies of effort, such as consolidating computer support, comptroller functions and possibly supply support and capital equipment management. The guidelines for this consolidation are that there be minimal personnel turbulence. WRAIR had a bad experience from a reduction in force dictated by the actions of Congress a few years ago, and although only a few people were critically affected when it was all over, the effect on morale was Institute-wide and catastrophic. We want to avoid the stress that results when people do not know what is going to happen to them. The understanding is that if we intend to make changes in staff, we will use attrition as a mechanism for separating people. We will not be throwing people

out of their jobs nor discontinuing jobs. The whole objective is not to bring about a reduction in the level of support of research, but to improve its efficiency. We have been operating with some duplication of effort between the Institutes, and it seems to us that by consolidation we should be able to enhance efficiency and get more out of the resources.

Another topic is Rocky Mountain spotted fever vaccine development effort. For the last 18 mon we have been evaluating the vaccine in man with NIAID funding support. A third phase of human testing began in August and now is in the data collection and analysis phase. NIAID is proposing to sponsor an additional study of this vaccine under contract with the University of Maryland, in which the subjects will be challenged with infectious organisms after vaccination. We will be collaborating to support this effort if it goes as planned. Our support would include access to investigational new drug data, provision of sufficient vaccine for the trial, and additional split-specimen laboratory determinations so that the data accumulated in the new trial can be linked to the data already on hand. This is important because the present bank of information is not all that large. And later, if the vaccine turns out to be as promising as we hope it will be, there may be a need to produce a much larger quantity of vaccine. The remaining supply of vaccine is limited. We may join with NIAID to support production of a larger lot of vaccine which will meet national needs for a significant period of time.

Patient Care Agreements. We reached an agreement during the year with the Center for Disease Control (CDC) by which we would care for any of their people exposed do high-hazard agents. The agreement had just been completed when 2 staff members of CDC were exposed to LAS. They were transported here by Air Force air evacuation, placed in our isolation suite and given gamma globulin. The aircraft landed at Hagerstown; the patients were then moved by ground ambulance to USAMRIID. By clinical course and serological data they were judged not to have been infected, and they went home on day 21. We hardly had the suite decontaminated when a Peace Corps worker from Senegal arrived at WRAMC, having been evacuated with an illness that included fever and myalgia and a good deal of uncertainty. We admitted him here primarily for the purpose of ruling out LAS from which he could have been convalescing. Apparently LAS was not his illness. If he had an established diagnosis, it might have been typhoid fever. The critical specimens had been examined before he left Africa. We did not confirm any diagnosis here.

We have entered a similar agreement with the Department of Agriculture, animal disease, high-hazard containment laboratory at Plum Island, just off Long Island. We have agreed to accept their patients suspected of having RVF or any other disease of high-hazard.

In support of these commitments, the ability to move a patient in a decent state of biohazard containment is a requirement. The Air Force is interested in acquiring Vickers isolators and will probably do some joint evaluation with us. The Canadians have these isolators and have conducted transoceanic exercises with simulated patients between Toronto and Germany and back again. I think they have successfully established that long-haul movement of patients, at least no critically ill patients, is feasible.

Swiftwater Facility. During the year, the management of the organization has all changed. Merrell-National, which operated the facility for us, decided to get out of the biologics production business and gave their entire facility (much more than the Army portion of it) to the Salk Institute of Loyola. The Salk Institute formed a government operations division and continued to contract and operate for the Army our biologics production suite. For other reasons, the Salk Institute sold the balance of the Swiftwater plant, which includes a large influenza vaccine, yellow fever vaccine and DPT production units to Connaught Laboratories of Canada. The staff serving the Army contract remained essentially the same, and the program goes on vigorously. There have been a few problems sorting out our product liability. At first, it was thought that this insurance for Salk would be ridiculously expensive. Later, a source from which to purchase adequate liability insurance was located. Vaccine production is moving along without difficulty.

The National Academy of Science/National Research Council (NAS/NRC) is establishing on our behalf a committee on the effects of multiple immunizations. We requested this effort and are funding it because of our concerns, and our recognition of the concerns of others, about the health effects of repeated immunizations over a significant portion of a life-span to large numbers of agents. The population of workers at Fort Detrick was immunized extensively and is now being studied by Drs. Mason and Hoover of the National Cancer Institute. They first conducted a pilot study consisting of health follow-up of 100 persons. This has been completed and has encouraged them to consider the entire population. They are now at the stage of having distributed an initial questionnaire and have made a second mail approach to the nonresponders. During the course of this investigation, I am sure we will get an updated briefing on the subject. One of the things that prompted our request to NAS/NRC was the fact that if you read the recommendations of the advisory committee on immunization to the CDC, you will see occasional phrases like "since the safety of repeated immunizations has not been established" or other references of that sort. There is concern that there might be adverse health effects. What we need from the committee is an assessment of where we are, and what information is available. If there are health effects we should look for, we may need some assistance in designing prospective studies. As the USAMRIID program produces results, there will be the option to immunize the Armed Forces to more diseases. If there are adverse health effects that can be defined, we need to make them very clear.

Smallpox. If you have been following the comments in Science, you noticed that we received some publicity which was a little distorted; that has been taken care of. Essentially, there is a World Health Assembly plan for storage, research and diagnostic study of smallpox, which would reduce the number of smallpox laboratories to 5; maybe today it is down to 2 with the US and the USSR being the only ones still active. Japan has suspended its research on the disease, until it gets a better laboratory. They have moved all of their virus cultures to CDC this week for safe holding. The UK posture is not clear at this time. The Netherlands may be less interested than it was in keeping its activity going. At any rate, for the US there has been a series of joint planning meetings, involving DOD, CDC, American Type Culture Collection (ATCC), all of whom have smallpox virus, the Office

of International Health and DHEW. We have proposed a plan which is now ready for staffing with the decision makers in DHEW, ATCC and DOD. This plan will meet national needs for an adequate defense for smallpox should it be reintroduced, whether accidentally, naturally or deliberately. At the same time, the plan will allow compliance with the World Health Assembly proposal and will support the credibility of the US. Some of the elements of the plan are as follows: (a) DOD will continue indefinitely the vaccination of military forces as recommended by the Armed Forces Epidemiological Board; (b) the CDC will maintain its current diagnostic and virus identification and characterization research capability for the indefinite future and will invite members of DOD to join research there as desired (we may be interested in deter-mining the effects of ribavirin against smallpox in animal models); and (c) CDC will maintain current stockpiles of vaccine, which will be split between 2 locations in order to minimize the chance of catastrophic loss. The size of the stockpile is estimated to be 20 million doses or more. The DOD and CDC plan to maintain a vaccine immune globulin stockpile of a modest size. DOD has procured 5,000 vials, which represent the total national supply. This gives ethical support to the plan for continuing military vaccination. If we have adverse effects from immunization, either in a military vaccinee or a member of his family, we can do something about it. Furthermore, immune globulin would be good to have if smallpox suddenly appeared in this country, since it has an ameliorating effect if given after exposure. CDC will provide archival storage, again in 2 locations in and around Atlanta, for their own stocks of virus and for those received from DOD and ATCC. Both storage sites will have liquid nitrogen storage, biohazard containment and security provisions. CDC will notify all parties in advance of any plans to change the level of stocks. We think this plan will be approved and will settle the issue.

Laboratory Safety. The issue of safety has been of great concern to us for the entire year. We had an iodine isotope spill that involved 2 persons and was contributed to by an unrecognized insuffient air flow from a hood. We have taken the necessary steps to inprove isotope containment. We have installed improved monitoring that uses activated carbon filters to trap iodine; this also uses a sniffer near the breathing zone of the persons at risk, to determine what level they have been exposed to during the procedure. We feel we now meet good practices regarding isotope containment.

Monkey Bites. We continue to be concerned about monkey bites and the hazard they present to the staff. We had one exposure this year in which the biting monkey was found to have oral lesions. It was impossible to exclude simian B virus exposure to the individual, so he was given the immune material. We are going to look at the possibility of picking up the Eli Lilly herpes B vaccine and bringing it to the state where it will be available to laboratory workers at risk throughout the US and elsewhere. Lilly carried the vaccine to the IND stage, but never used it for other than their own staff. The vaccine they made is no longer available. It became outdated and was destroyed. Lilly is willing to provide us access to their data and to assist in any arrangement we might make to develop a new supply.

Tentative Concept for BW Medical Defense. I would like now to show you a concept I'm getting ready to use for nonmedical decision makers of the Army about the posture of the nation for BW medical defense. Table IV shows the preliminary line-up of the concepts.

TABLE IV. COMPONENTS OF MEDICAL DEFENSE AGAINST BW

1. Agent detection
2. Case recognition*
3. Agent identification and characterization*
4. Epidemiological investigation
5. Prevention
   Transmission interruption; passive immunization*; active immunization*; chemoprophylaxis*
6. Treatment*

*USAMRIID efforts.

I would propose that these are some of the critical components of a system for medical defense. I would appreciate feedback during the course of the meeting. We make a contribution in certain areas. Agent detection is the responsibility of DARCOM working in the Chemical Systems Laboratories at Edgewood Arsenal. They have an agent-detecting machine well along in development with a target date of just a few years before being ready for deployment. We in the Medical Department have a major problem to design an effective interface with the machine. Some element must take the sample it collects and decide whether or not a BW agent is involved, and if so, which one. Obviously, the epidemiological capability is well-established at WRAMC, and we will not duplicate it. I mentioned earlier that we might get some entomology capability here for arbovirus research, so we may be able to place an asterisk after transmission interruption by this time next year.

The first posture of preparation is Technology Stockpile (Table V), i.e., to develop a medical defense but not really deploy it. This is the posture we are in for a large number of agents: Q fever, VEE, tularemia,

TABLE V. POSTURE I: TECHNOLOGY STOCKPILE

| | |
|---|---|
| Vaccines | Developed<br>Tested<br>Trial lots produced<br>Production cookbooks written<br>At-risk laboratory workers use vaccine<br>Stored in bulk in R&D laboratory |
| Diagnostic methodology | Established at research laboratory level |
| Medical forces | Minimal awareness of BW |
| Force readiness lead-time | Months to years |

Current posture for many agents

botulism, eastern and western equine encephalitis, Chikungunya, RVF and probably others; we have made at least one effective vaccine for each of these dis-eases. We have production know-how and the ability to diagnose them, at least at the research laboratory level at USAMRIID. Nobody in the Medical Departments of the Armed Forces is aware of what we have and what we are doing to correct this. The presence here of Colonel Giddens from the

Academy of Health Sciences and Colonel Robert Cutting from DARCOM represents two efforts to improve that awareness. Under this concept, if we really wanted to get the Armed Forces of the US ready after we knew we needed to, it would take months or years. In 1971, when the VEE episode occurred, it took a fair number of weeks to convert a bulk, frozen vaccine to a repackaged ready-to-use vaccine, move it to the field, and inject it into animals that needed it. The RVF outbreak, which will be discussed later, will also illustrate a production lead-time of several months after the decision is made.

Table VI presents a "more ready" posture, Forward Positioning. For this posture we would make enough vaccine for the total force and would position enough diluted vaccine in forward depots in Army Europe or Korea to take care of needs. You would still have an irreducible lead-time for the immune response and the time it takes to distribute and inject vaccine into large numbers of people. So, after you know you needed the vaccine, it would take weeks before protection was achieved. Any scenario of conflict for Army Europe would consider this unacceptably late. People have said that the next war in Europe would be decided in 1, 2, 3 or 4 weeks. Certainly, if we want to change our posture from where we are now, it is going to take a lot of intensive work and time to get there.

TABLE VI. POSTURE II: FORWARD POSITIONING

| | |
|---|---|
| Vaccines | Developed and tested<br>Total force quantity produced<br>Stored at theater depots, diluted in dispensing containers<br>Ready quantity<br>Injection planned, equipment stocked<br>Reserves stored in bulk<br>Replacement production planned |
| Diagnostic methodology | Special reagents and equipment at theater depots<br>Technicians trained in use during schooling |
| Treatment | Concepts in AMEDD emergency plans<br>Stress on therapy of infection acquired by the respiratory route<br>Tested under simulated trials |
| Medical forces | Senior planners alerted and informed |
| Force readiness lead-time | Weeks |
| Time to achieve | 1-3 years, depending on resources applied, for those agents for which vaccines are at human testing stage. Others variably longer |

Table VII presents an even higher degree of readiness, in which vaccines are moved out to the dispensary so that the morning you decide to use the vaccine, immunization can be started. The high-value forces, the

strategic forces, major command and control units, would be already immunized. Plans would be available to carry out the balance of the immunization in a hurry. Replacement production and back-up stocks would be in these plans. With this level of posture, some people, including medical staff, would already be protected. We are in this mode now for yellow fever and plague. We immunize the "ready forces" and boost them only when there is a requirement.

TABLE VII. POSTURE III: SELECTIVE DEPLOYMENT

| | |
|---|---|
| Vaccines | Stocks at unit level in dispensing containers<br>High priority forces already immunized for selected agents<br>Exercised-plans and equipment for immunizing all forces<br>Reserves stored in bulk<br>Replacement production planned |
| Diagnostic methodology | Special reagents and equipment set up in medical units<br>Technicians trained on coded unknowns periodically |
| Treatment | Plans fully developed and exercised periodically |
| Medical forces | All echelons alerted and informed |
| Force readiness lead-time | Days |
| Time to achieve | 5 years? |

Table VIII presents a battle-ready posture. Everyone is immunized and boosted as required. Stocks and reserves would already be on hand and at key points. Diagnostic and treatment plans would have been optimized for those personnel who had not been immunized. This level of readiness would take a long, long time to achieve. If we were in this mode, we would be ready for a BW attack. There are obvious increased costs as you move from one level of readiness to a higher one. It would cost a lot to get there and costs would be high to maintain vaccine stocks at a realistic level of readiness. The health effects of keeping a large population immunized needs to be assessed as a subcomponent of this posture.

I solicit your comments on these concepts.

TABLE VIII. POSTURE IV: BATTLE READY

| | |
|---|---|
| Vaccines | Forces all immunized<br>Boosted periodically<br>Stocks in dispensing container with replacement production ongoing<br>Reserves in bulk<br>Replacement production planned |
| Diagnostic methodology | Medical units equipped<br>Reagents on hand<br>Personnel trained<br>Exercises frequently with coded unknowns |
| Treatment | Plans challenged frequently by unannounced simulations |
| Medical forces | Ready |
| Force readiness lead-time | None |
| Time to achieve | More than 5 years |

Lastly, I would like to bring to your attention the Institute's updated research goals, listing the agents we are studying. They are in the handouts. Table IX presents the list of diseases we are trying to stay aware of, while not actually conducting research on them here at USAMRIID. Some of the research is being done at WRAIR and at other agencies; the Navy, for instance, is taking a look at coccidioidomycosis.

TABLE IX. DISEASE RESEARCH GOALS

| | |
|---|---|
| PRIORITY I | |
| Viruses | Lassa, Ebola, Marburg, Bolivian hemorrhagic fever (Machupo), Argentine hemorrhagic fever (Junin), Korean hemorrhagic fever, Rift Valley fever, Congo/Crimean hemorrhagic fever |
| Toxins | Botulism, anthrax |
| Bacteria | Legionnaires' disease bacillus |
| PRIORITY II | |
| Viruses | Chikungunya, dengue-1, tick-borne encephalitis (RSSE, OHF, KFD), Japanese B encephalitis, VEE |
| Toxins | Staphylococcal enterotoxins, *Pseudomonas* exotoxins |
| Rickettsiae | Q fever (*C. burnetii*), North American tick typhus (Rocky Mountain spotted fever), exotic spotted fevers, epidemic typhus |

| | |
|---|---|
| PRIORITY III | |
| Viruses | Eastern and western equine encephalitis, yellow fever, smallpox |
| Bacteria | Melioidosis, tularemia |
| Combined injury | Radiation and infection, chemical agent and infection |
| MONITORED AGENTS | |
| Viruses | Rabies, dengue-2, -3, -4, O'nyong-nyong, influenza |
| Bacteria | Plague, glanders, typhoid fever |
| Chlamydia | Psittacosis |
| Fungi | Coccidioidomycosis |
| Modified agents | Recombinants, antibiotic-resistant strains |
| Toxins | Cholera, Shigella, diphtheria, marine |

DISCUSSION

Dr. Woodward: With the 2-year limitation, will it be possible for you to retain members as consultants?

Colonel Barquist: Yes, it is actually a 4-year limitation. The initial appointment is for 2 years. This is an appointment to a particular subcommittee; and my understanding is that it need not affect a person's consultant status at all. One must, however, be a consultant to receive formal appointment to a subcommittee.

Dr. Woodward: You have answered the point which I was concerned about, since you need to maintain continuity.

Colonel Barquist: Is there anyone from MRDC staff who would like to address this area?

Dr. Noyes: It is correct that individuals can be consultants for more than 4 years, but are limited to 2 consecutive 2-year terms on a subcommittee or study group.

# RIFT VALLEY FEVER (RVF) RESEARCH - THE EGYPTIAN OUTBREAK AND USAMRIID RESEARCH APPROACHES TO RVF

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Rift Valley fever (RVF) virus was discovered in the 1930s while investigating an explosive epizootic of sheep. Findlay and co-workers studied the pathogenesis of RVF infections in the Wellcome Laboratories in the 1930s (1) and Mims added elegant quantitative observations of the disease in mice in the 1950s (2-6). Although several other laboratories, particularly in South Africa, contributed to RVF studies the only other concerted effort came from the US Army, which resulted in quantitative studies on several animal infections, definition of the aerosol threat, and production of a safe and efficacious formalinized human vaccine. RVF research was relatively inactive for several years: the disease was benign in humans, a veterinary vaccine had been produced, the affected area was defined, and work with the virus in developed countries carried the risk of introduction of the disease. Two new developments have changed our perception. In 1975 the South Africans recognized the occurrence of fatal hemorrhagic fever and encephalitis in association with RVF infection. Then in 1977 these complications were confirmed during an epidemic in Egypt. More importantly the Egyptian epidemic represented an extension of the disease into new territory. Egypt may occupy a key role in dissemination of African diseases to the Mediterranean basin, as evidenced by the spread of African horse sickness.

Thus several features of RVF contributed to our decision to work with the virus: the changing epidemiology and clinical picture; the virus requires specialized containment facilities which are available at USAMRIID, but which limit work with the virus in the United States. In spite of the need for containment to protect the environment from RVF introduction, personnel can be protected with vaccine so that cumbersome Class III cabinet or "space suit" technology need not be used. The virus itself grows to high titer, is stable, and plaques readily simplifying in vitro manipulations. We are not working intensively with other Bunyaviruses so RVF viruses provide a prototype of this increasingly important family of viruses. RVF, like other Bunyaviruses, possesses a segmented genome, thus increasing its potential for recombination; this may have important consequences for natural or laboratory variation. Finally the infection is lethal for adult animals of several species, which increases its utility in vaccine or chemotherapy studies.

RVF was first recognized in Egypt near Zagazig, Sharqiya province in the summer of 1977 (Fig. 1). The disease spread to Qalyub and Giza provinces. Exact figures are unknown, but about 200,000 human cases probably occurred. About 1% of these cases were complicated by severe encephalitis or fatal hemorrhagic fever. Extensive sheep and lamb mortality occurred. The disease was also confirmed in Aswan, Lower Egypt. Objective data do not permit exact interpretation but the disease in Upper Egypt began about the same time as disease in Lower Egypt. One commonly voiced hypothesis is that a reported 1976 epidemic in the Sudan spread to contiguous areas in Upper Egypt and later was introduced into Sharqiya by clandestine camel caravans. During the winter of 1977 no transmission of RVF was recorded in Sharqiya,

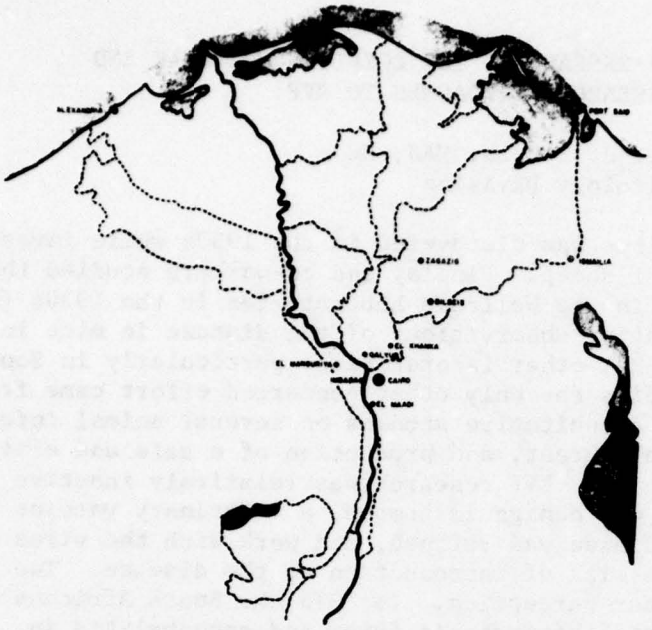

Fig. 1. Map of northern Egypt

although some disease continued to occur in Upper Egypt. The disease survived the winter in Egypt, so that during the summer of 1978 there were new deaths in animals and humans. The exact number is once again unknown. NAMRU-3 (Cairo) has, however, documented extension of disease in 1978 into areas where no history of disease or serologic evidence of virus activity existed in 1977.

Classical concepts of epidemiology in the Rift Valley including South Africa are based on an ill-understood sylvatic cycle, presumably through mosquitoes. The reservoir is unknown, perhaps ungulates, rodents, or vertically infected arthropods. Sentinel animals at the forest edge may be infected. During a particularly rainy summer with expansion of arthropod populations, sheep are infected and serve as amplifiers and the major economic target of infection. Virtually all lambs or lamb fetuses are lost and about one-fourth of adult sheep die. The disease is usually considered to be mosquito-transmitted. Man is thought to be infected primarily by aerosol or contact, when animals are slaughtered or autopsied or when raw meat is ingested. It is difficult to assess mosquito-to-man transmission in this setting.

The Egyptian epidemic differed in several aspects. Camels, water buffalo and other domestic animals lived near sheep and presumably participated in the infectious cycle. Man himself was present in considerable numbers and sustained high viremias so the humans may have interacted with arthropods as target or amplifier. The epidemic was presumed to be mosquito-borne, although there were no isolates from unengorged mosquitoes, even Culex pipiens, the major species represented in all collections.

RVF infection of the human presents as an acute undifferentiated febrile illness (Table I). Since the 1950s ocular complications have been noted. Only since the 1975 South African experience have encephalitis and hemorrhagic fever been attributed to RVF. Encephalitis occurs as a late complication and usually is noted as the acute disease wanes. There are no virus isolations from these patients but rising antibody titers confirm recent infection. Hemorrhagic fever is usually seen during the evolution of the acute infection. Occasionally epistaxis occurs during an uncomplicated case, but when generalized hemorrhagic manifestations and icterus are seen, a fatal outcome is the rule. The limited observations to date suggest that both peripheral capillary damage and massive hepatic necrosis contribute to the fatal outcome. An estimated 1% of human RVF cases in Egypt were complicated by encephalitis or hemorrhagic fever.

TABLE 1. RVF IN HUMANS

| |
|---|
| Acute, undifferentiated, febrile illness comprises 99% of cases |
| 3-7 day incubation |
| Fever, headache, myalgia, malaise |
| Duration usually 3-5 days |
| Possibly 1/4 of cases subclinical |
| 3/4 viremic when first seen |
| Clinical attack rates may exceed 70% |
| Ocular complications |
| Retinal macular lesions |
| Usually noticed as acute illness fades |
| Antibody-positive |
| Transient or permanent |
| Encephalitis |
| Described in South Africa (1975) and Egypt (1977) |
| May be severe and even fatal |
| Residua common |
| Usually noticed as acute disease wanes |
| Antibody-positive |
| Hemorrhagic fever |
| Described in South Africa (1975) and Egypt (1977) |
| Occurs as acute disease evolves |
| Deaths day 7-10 |
| Viremia common |
| Generalized hemorrhage and icterus |
| Massive liver necrosis at autopsy |
| Schistosoma mansoni common in Egyptian fatalities |

Results of our initial studies on the pathogenesis of RVF in laboratory rodents are presented in Table II. The strain of virus used, Zagazig 501, was isolated by Dr. James Meegan (NAMRU-3) from a fatal case of hemorrhagic

fever in 1977. Disease in these laboratory hosts fell into 2 broad categories. Hamsters, rats, and mice usually died within 3-5 days of inoculation with fulminant liver necrosis. They were also very sensitive to infection, the $LD_{50}$ for the 3 species were approximately 0.1, 10, and 50 PFU, respectively. Cotton rats (Sigmodon hispidus), Calomys callosus, and the gerbil are equally sensitive to infection, since antibodies are found in convalescent sera from animals receiving only 10 PFU of virus. However, even $10^7$ PFU fail to kill half the animals. Equally important is the pattern of disease. Most deaths occur in the second week of illness and are associated with the presence of serum antibody. Clinical and histopathological examination confirmed the presence of encephalitis with virtually no liver abnormalities.

TABLE II. SUBCUTANEOUS INOCULATION OF ZAGAZIG 501, $FRhL_2$

| Animal | Cause of death | $Log_{10}$ PFU | Survivors/ Inoculated | Antibody +/ Total |
|---|---|---|---|---|
| Syrian hamster | Hepatic necrosis | 1 | 0/10 | - |
| Sprague Dawley rat | Hepatic necrosis | 1 | 6/15 | 3/ 6 |
| | | 3 | 3/25 | 2/ 2 |
| | | 5 | 1/21 | 1/ 1 |
| ICR mouse | Hepatic necrosis | 1 | 10/10 | 0/10 |
| | | 3 | 4/14 | 2/ 4 |
| | | 5 | 0/10 | - |
| Cotton rat | Encephalitis | 1 | 5/ 5 | 5/ 5 |
| | | 3 | 5/ 5 | 5/ 5 |
| | | 5 | 5/ 5 | 5/ 5 |
| | | 7 | 11/19 | - |
| C. callosus | Encephalitis | 1 | 8/ 8 | 3/ 4 |
| | | 3 | 10/12 | 2/ 3 |
| | | 5 | 11/12 | 7/ 7 |
| | | 7 | 10/12 | - |
| Gerbil | Encephalitis | 5 | 6/10 | 2/ 2 |
| | | 7 | 11/20 | 2/ 2 |
| Guinea pig | --- | 1 | 5/ 5 | 0/ 5 |
| | | 3 | 5/ 5 | 1/ 5 |
| | | 5 | 10/10 | 5/ 5 |
| | | 7 | 12/12 | - |

Figure 2 shows the early death and progressive viremia seen in hamsters, the purest infection of this type. The encephalitic pattern is shown in Figure 3 in cotton rats. Although respectable serum virus concentrations of

$10^4$-$10^5$ PFU/ml are present on day 1, virus is scarcely detectable by day 2, and low levels of antibody are present by day 3.

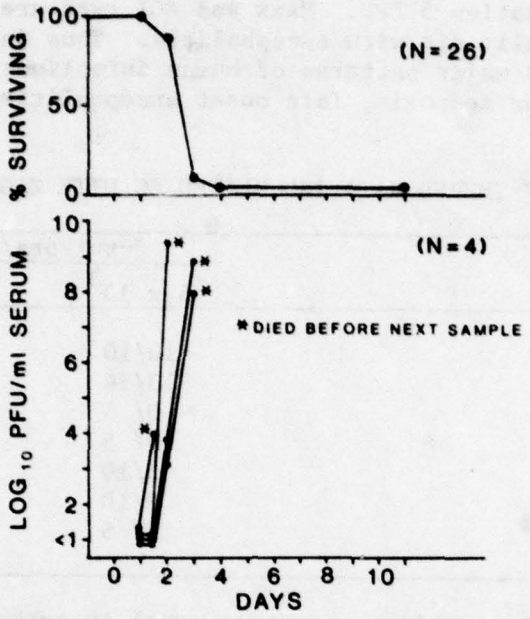


Fig. 2. RVF in Syrian hamsters (10 PFU, SC)

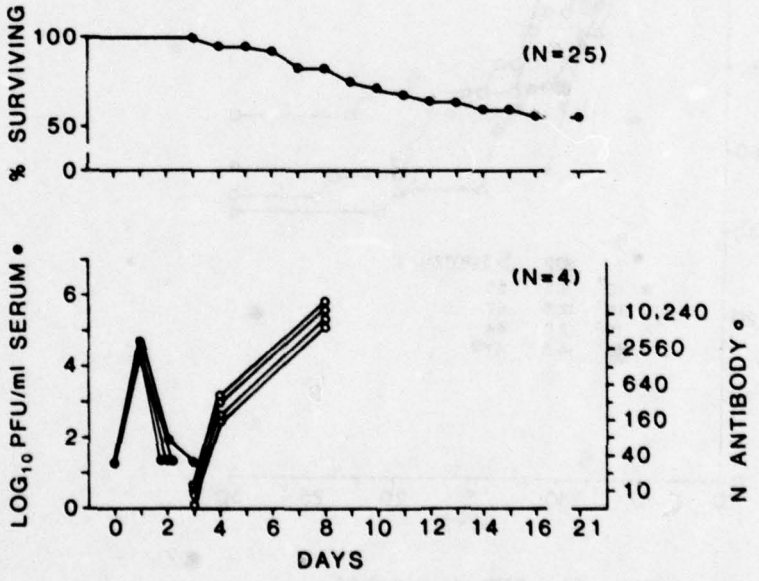


Fig. 3. RVF in cotton rats ($10^7$ PFU, SC)

Host genotype within a species is an important determinant of the outcome of infection as exemplified by the results of SC inoculation of 7 inbred rat strains (Table III). The $LD_{50}$ for resistant Lewis rats is $>5 \times 10^6$ PFU. Wistar Furth or Brown Norway rats succumb to liver necrosis within 4 days of inoculation 5 PFU. Maxx and ACI rats are intermediate in susceptibility and usually die with encephalitis. Thus in one species we have analogues of the 3 major patterns of human infection: death with fulminant disease and liver necrosis, late onset encephalitis and nonfatal infection.

TABLE III. SURVIVAL OF INBRED RATS INOCULATED SC WITH ZAGAZIG 501

| Rat strain | Survivors/Inoculated | |
|---|---|---|
| | $5 \times 10^3$ | $5 \times 10^5$ |
| Lewis | 10/10 | 10/10 |
| F 344 | 13/14 | 17/20 |
| Wistar Furth | 0/ 5 | 0/ 5 |
| Brown Norway | 0/ 5 | 0/ 5 |
| Maxx | 5/10 | 6/10 |
| ACI | 9/10 | 5/10 |
| Buffalo | 5/ 5 | 9/10 |

The gerbil probably provides a genetic model in outbred animals (Fig. 4). Regardless of the virus dose inoculated from $10^1$-$10^7$ PFU, 44-67% of the animals died, although the median time-to-death was less with higher

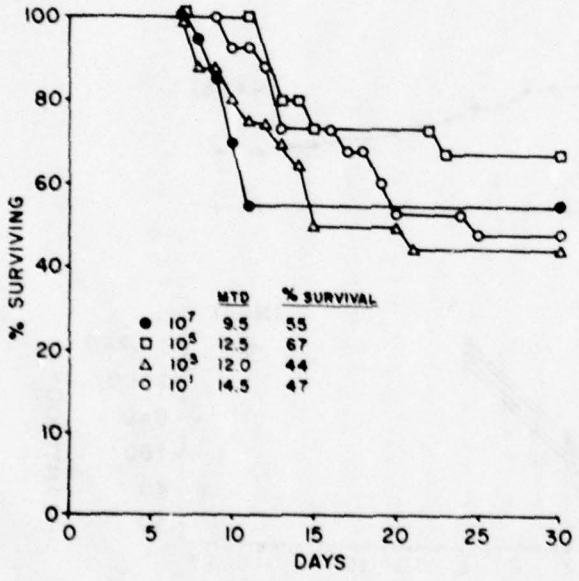


Fig. 5. RVF in gerbils

doses. This suggests that about half the gerbils tested were susceptible to any dose of virus (no matter how small) but that the remainder could

resist the largest dose tested. Appropriate experiments are underway to establish the genetic basis for this phenomenon.

The genetic argument applies not only to the infected host but also to the virus strain. Three other RVF isolates from disparate sources were tested quantitatively in all the species previously inoculated with the Zagazig 501 strain. Patterns of mortality were similar except for rats. Serologically indistinguishable South African isolates from 1951 and 1975 and a high-passage Ugandan isolate required more than a million times as many PFU to kill outbred Sprague Dawley rats as did Zagazig 501. Three other Egyptian isolates were also lethal for rats, confirming this property as a biological marker for viruses from the Egyptian epidemic rather than a unique feature of the Zagazig 501 hemorrhagic fever isolate.

Several short term goals are being attacked. Serial sacrifice studies with determination of organ virus titers and immunofluorescent antigen distribution should help us understand how viremia is terminated in resistant animals, how the liver, is protected, and what is the pathogenesis of the encephalitis. Breeding experiments are underway to define the formal pattern of host resistance and its relation to the major histocompatibility complex. Pathogenetic and genetic studies should then come together to elucidate the mechanism of host genetic resistance. We would also like to develop a primate hemorrhagic fever model. We plan to apply the technique of RNA fingerprinting to study the evaluation and epidemiology of individual genome segments.

More tentative, long-term goals include hopes to correlate eventually animal genetic data with the human situation through in vitro testing. We have spoken very little about the role of virus genetics compared to that of host genetics but this must be explored further. The rat pathogenicity of Egyptian strains provides one possible approach. Published work also suggests that unselected clones (7) or clones derived under selective pressure (8) may be useful as well. We plan to exploit the newly developed hybridoma technology to provide monoclonal antibodies with their many potential uses.

## LITERATURE CITED

1. Findlay, G. M. 1932. Rift Valley fever on enzootic hepatitis. Trans. R. Soc. Med. Hyg. 25:229-265.

2. Mims, C. A. 1956. Rift Valley fever virus in mice. I. General features of the infection. Br. J. Exp. Pathol. 37:99-109.

3. Mims, C. A. 1956. Rift Valley fever virus in mice. II. Adsorption and multiplication of virus. Br. J. Exp. Pathol. 37:110-119.

4. Mims, C. A. 1956. Rift Valley fever virus in mice. III. Further quantitative features of the infective process. Br. J. Exp. Pathol. 37:120-128.

5. Mims, C. A. 1956. Rift Valley fever virus in mice. IV. Incomplete virus; its production and properties. Br. J. Exp. Pathol. 37:129-143.

6. Mims, C. A., and P. J. Mason. 1956. Rift Valley fever virus in mice. V. The properties of a haemagglutinin present in infective serum. Br. J. Exp. Pathol. 37:423-433.

7. Boyle, J. J. May 1966. Some characteristics of plaque variants of Rift Valley fever virus. Technical Memorandum 297, Fort Detrick, Frederick, MD (AD482 585).

8. Smithburn, K. C. 1949. Rift Valley fever: the neurotropic adaptation of virus and experimental use of this modified virus as a vaccine. Br. J. Exp. Pathol. 30:1-16.

## DISCUSSION

Dr. Nathanson: What about encephalitis in the host? You did not say anything about titers in the brain.

Major Peters: We've had very erratic results and I'm concerned about the technical aspects of the assay. There is usually virus present in the brain and $10^5$ PFU/gm of tissue represents a typical concentration. I would like to do more tests in rats, in order to be more certain of the assay.

Dr. Nathanson: Do you have an immunoassay stain?

Major Peters: No, but it has a high priority.

Dr. Pappenheimer: Have you tested the F1 rat?

Major Peters: We examined 2 F1 crosses, resistant Lewis x susceptible Brown Norway and resistant Lewis x susceptible Wistar Furth. Both are resistant.

Dr. Woodward: This is a very nice report. Any information on the shelf-life of the vaccine and its protectivity?

Major Peters: Dr. Cole will discuss the production of vaccine; however, the original vaccine was prepared in 1968 and has not diminished in its mouse potency as assayed over the years. The vaccine is lyophilized and has been stored at -20°C. In terms of human immunogenicity, we do not yet have a carefully laid-out study, but the neutralizing antibody data indicate no apparent decrease. We assume that there has been no loss in potency, because of animal data and the fact that we have had no RVF infections among our laboratory personnel.

Dr. Johnson: What is the USDA doing about vaccinating animals in this country and elsewhere? Wherever the need for animal vaccination might arise?

Major Peters: USDA has plans to establish diagnostic facilities at Plum Island. They have vaccinated their personnel and are sending 2 people here for training next week. I can not comment on their vaccination plans for animals. I have told them that we do not regard our human vaccine as a

stockpile for agricultural use. They are planning to test our vaccine in sheep and cattle, but I don't know the rationale for this testing. Most likely, it is to determine if a formalin-killed vaccine is immunogenic.

Dr. Johnson: I recall the VEE outbreak and this serves as an excellent model. The potential for RVF outbreaks in several parts of the world represents an even greater risk than VEE. It seems to me that existing vaccine supplies would quickly be drained. I am not criticizing your program, but I did want to know the USDA plans.

Colonel Barquist: I share your perception of the problem and we have tried to do something. I have contacted General Tom Borne, Chief of the Veterinary Corps, U.S. Army, who was intimately involved in VEE outbreaks; he is well aware of the problem. He will get in touch with Dr. Mohern, who has parallel responsibilities in USDA. This meeting has not occurred, but is still planned. We hope to make the point that our vaccine is for humans; we must interest them in acquiring a vaccine capability for animals. The problem now is whether vaccine or slaughter represents the best approach to control an RVF epidemic. USDA personnel are working on this problem and seem to be shifting to the point of considering vaccination as an approach.

Dr. Sanford: What is the availability of the agricultural vaccine from Kenya or South Africa?

Major Peters: The South Africans, as I understand it, are capable of producing several tens of thousands of doses/month if they are tooled up. Some countries in the Middle East are purchasing vaccine from them. This vaccine seems to be effective. It is formalin-inactivated, produced in baby hamster kidney cells, has a high titer, and is a good antigen. The Kenya vaccine is a live, attenuated vaccine, is abortogenic, and has not been characterized sufficiently to regard it as a reasonable vaccine.

Dr. Woodward: About 3 years ago there was a threat in South Africa and Dr. James Gear wired us to see if he could obtain some of our vaccine. Colonel Barquist was instrumental in having the vaccine sent through the State Department. What happened to your own titer, Dr. Peters, before and after your Egyptian experience?

Major Peters: I sort of acquired my titer on the way to Egypt. Somewhere at Dulles Airport, I probably started making antibodies. I do not like to think I was infected. I had antibodies on my return and no clinical illness while there.

Dr. Rammelcamp: Could you elaborate on infections by the aerosol route?

Major Peters: The aerosol data generated by the Biological Laboratories, Fort Detrick, showed that RVF virus is highly infectious for experimental animals, including primates. Anecdotal evidence in the field also suggests that it is infectious for man by aerosol. During the Egyptian experience, members of a field team became infected 3 days after slaughter of a sick sheep during which blood was sprayed over all of the room. The sheep blood was later tested and found to have a titer of about $10^9$ $LD_{50}$/ml.

Dr. Nathanson: How about recombinants between RVF and other Bunyaviruses? Has it been done?

Major Peters: We do not known what the restrictions on recombinants between different Bunyaviruses will be. We know that between closely related B viruses, such as LaCrosse and snow shoe hare, Dr. Dave Bishop has been able to multiply-infect cells and generate recombinants, or reassortment (or whatever) with high frequency. We do not know what will happen as we move away from closely related viruses. We are interested in this approach and know that it represents a formidable amount of resources, time, mutants, and markers.

# RIFT VALLEY FEVER VIRION CHARACTERISTICS

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Virology Division

Rift Valley fever is a proposed member of the Bunyaviradae family, which is the largest family of viruses and is composed of 2 groups: the Bunyavirus genus consisting of 87 serologically interrelated viruses and another 75 viruses which are morphologically similar, but unrelated, and referred to as Bunya-like viruses.

The Bunyavirus genus, or more commonly called the Bunyamwera supergroup consists of 11 serologically related groups (Table I). There are individual viruses within each serologic group which cross-react with viruses of other groups, within the genus. The smaller groups, like Bwamba and Patois, are usually limited to specific geographical areas, the former being found in Africa and the latter in North America. The larger groups, like Bunyamwera and California, generally have a much larger geographic distribution with species from most continents. Most of these viruses are mosquito-borne; however, members of Guama and Simbu groups are known to be transmitted by Phlebotomus spp., as well as Culicoides spp. Hosts for these viruses include numerous rodents, marsupials, lagomorphs, bats, various birds, domestic animals, primates and man. Members of the Bunyamwera, Guama and C groups can cause febrile illness in man. Members of the California group, such as LaCrosse, can cause encephalitic deaths in humans. Oropouche, a member of the Simbu group, has caused several epidemics in Brazil.

TABLE I. GROUPS OF THE BUNYAMWERA SUPERGROUP

| Group | No. of strains | Group | No. of strains |
|---|---|---|---|
| Bunyamwera | 18 | Koongol | 2 |
| Bwamba | 2 | Patois | 4 |
| C | 11 | Simbu | 16 |
| California | 11 | Tete | 4 |
| Capim | 6 | Unassigned members | 7 |
| Guama | 6 | | |

Dr. David Bishop, of the University of Alabama at Birmingham, as well as Drs. Obijeski and Trent of CDC have done biochemical studies on viruses within the California group. Biochemical studies have also been done on members of the Bunyamwera and C groups.

The second division of Bunyaviradae consists of serogroups of viruses which resemble those of the supergroup on electron micrographs (Table II). These viruses are related within their respective groups, but are not related between groups. I might emphasize that there is no serological relation between these viruses and any of the viruses of the supergroup. These viruses are also transmitted by mosquitoes, phlebotomine flies and ticks and have essentially the same host range as the supergroup. Many are known to cause disease in man. Dr. Bishop, under contract with WRAIR

TABLE II. GROUPS OF POSSIBLE BUNYAVIRUSES ANTIGENICALLY UNRELATED TO SUPERGROUP VIRUSES

| Group | No. of strains | Group | No. of strains |
|---|---|---|---|
| Uukuniemi | 7 | Kaisodi | 3 |
| Anopheles A | 3 | Mapputta | 3 |
| Anopheles B | 2 | Nairobi sheep disease | 2-3 |
| Bakau | 2 | Phlebotomus fever | 20 |
| Congo-Crimean hemorrhagic fever | 2 | Turlock | 3 |
| | | Unassigned viruses | 7 |

has initiated studies with the phlebotomus group to determine the structural protein and do the RNA finger printing for selected members, whose physical and morphologic characteristics have been studied. Uukuniemi has also been extensively analyzed biochemically. Congo-Crimean group of viruses are currently being studied at USAMRIID.

Rift Valley fever (RVF) virus falls into the unassigned group of viruses and is serologically unrelated to any other known virus.

Members of Bunyaviradae contain single-stranded RNA in 3 segments. This could allow for reassortment of genetic information when 2 different viruses coinfect the same cell. Dr. Bishop has been assessing how closely the viruses have to be related serologically for this to occur. Utilizing temperature-sensitive mutants he has shown that recombination occurs between members of the California group.

Virions within the family are spherical, enveloped, and approximately 90-100 nm in diameter. They consist of a unit membrane with surface projections which may be random or have an organized pattern.

When this project was begun, 4 isolates were considered. Table III lists these isolates and gives their passage histories. Entebbe (ENT) was

TABLE III. RIFT VALLEY FEVER ISOLATES

| Strain | Year isolated | Species | Passage history |
|---|---|---|---|
| ENT | 1944 | Eretmapodites pool (mosquitoes) | 184 mouse<br>1 FRhL-103 |
| SA-51 | 1951 | Sheep | 1 mouse, 3 sheep, 2 FRhL-103 |
| SA-75 | 1975 | Human | 3 FRhL-103 |
| Zagazig 501 | 1977 | Human | 2 FRhL-103 |

isolated in 1944 from a pool of mosquitoes in Uganda and has had 184 mouse IP or IV passages. At this Institute it was passed once in FRhL-103 tissue culture cells, which was then used for further studies. Entebbe is currently being used for vaccine production.

SA-51 was isolated from a sheep during an outbreak of RVF in South Africa in 1951, and has had 1 mouse and 3 sheep passages. We obtained some sheep serum at this Institute and passed it twice in FRhL-103 cells; second passage level was used for animal and tissue culture studies.

SA-75 was isolated from a febrile human case during a RVF outbreak in South Africa during 1975 and has been passed 3 times in FRhL-103 cells.

Zagazig 501 was isolated from a fatal hemorrhagic case of RVF during the 1977 outbreak in Eygpt. This sample has been passed twice in FRhL-103 cells; the second passage virus pool has been utilized for animal and tissue culture studies.

Table IV shows the number of plaque forming units (PFU) of virus/ml of tissue culture harvest from various established cell lines for the 4 isolates. All plaquing was done in 6- or 24-well plates utilizing Vero cells as the substrate.

Initially ENT was grown in the following cell lines: FRhL, Vero (monkey kidney), L929 (mouse fibroblast) and BHK (baby hamster kidney).

TABLE IV. RIFT VALLEY FEVER ISOLATES IN VARIOUS CELL LINES

| | PFU/ml IN: | | | |
|---|---|---|---|---|
| Strain | FRhL-103 | Vero | L929 | BHK |
| ENT | $7.5 \times 10^6$ | $2 \times 10^7$ | $4 \times 10^6$ | $3 \times 10^7$ |
| SA-51 | $2 \times 10^8$ | | | $1 \times 10^8$ |
| SA-75 | $2 \times 10^7$ | | | $3 \times 10^7$ |
| ZAG-501 | $2 \times 10^7$ | | | $6 \times 10^7$ |

The highest titers were obtained from Vero and BHK cells. At this time the decision was made to use BHK cells because they gave the highest titer and also because the majority of the information in the literature on structure of Bunyaviruses is based on BHK studies. When the other 3 isolates were received they were passed in the FRhL cells and then in BHK cells. The SA-51 isolate consistently produced 8 logs or better in BHK cells; therefore, it was chosen to do the initial physical and biochemical studies.

In Table V, some of the physical characteristics determined for the SA-51 isolate are compared with LaCrosse (LAC), a member of the supergroup, and Uukuniemi (UUK) a Bunya-like virus. The diameter of the viruses was measured from electron micrographs taken of negative stains

TABLE V. PHYSICAL CHARACTERISTICS OF RVF VIRUS, STRAIN SA-51

| Characteristic | UUK | LAC | SA-51 |
|---|---|---|---|
| Virus diameter (nm) | 114 | 91 | 89-130 |
| Density of virus in: | | | |
| Sucrose (gm/ml) | 1.17 | 1.18 | 1.19 |
| CsCl (gm/ml) | 1.20 | | 1.21 |
| Renographin (gm/ml) | | | 1.18 |
| Density of nucleocapsid in CsCl (gm/ml) | 1.31 | 1.31 | 1.29 |

from isopycnically-banded virus preparations. The average diameter was 100 nm (range 89-130). This compared favorably with reported values for LAC and UUK. The density of the intact virion was determined utilizing radiolabeled virus and spinning for 18-20 hr in a SW 50.1 rotor at 40,000 rpm. The gradients were fractionated and analyzed for radioactivity. The specific gravity was determined for peak fractions. To determine the density of the nucleocapsid, RVF virus was pretreated with NP-40, a detergent, to disrupt the outer protein coat which was separated by centrifugation. The density was determined to be 1.29 gm/ml.

The physical characteristics obtained for SA-51 isolate are in general agreement with reported values for the other members of the Bunyaviradae and therefore support the classification of RVF virus with this family.

Virus preparations utilized in morphologic studies were concentrated by direct pelleting with ultracentrifugation and purified by isopycnic banding in continuous renographin gradients. An aliquot of purified virus was mixed with 2-mercaptoethanol, sodium dodecylsulfate and appropriate buffers as described by Laemmli (1) and heated to approximately 100°C for about 20 min. The samples were mixed with a tracking dye and placed on cylindrical discontinuous gels which had previously been cast according to the method described by Laemmli. They were electrophoresed at 3 mA/gel until the tracking dye reached the bottom. The gels were fixed in 7.5% acetic acid overnight and the ones which had radiolabeled protein were frozen and sliced in 1-mm sections. Each slice was placed into a separate scintillation vial and assayed for radioactivity. Those gels containing nonradioactive protein were stained with Coomassie blue; the distances migrated were then calculated.

Figure 1 shows an electrophoretic profile of SA-51 labeled with $^{3}$H-amino acids on 8% polyacrylamide gels. The molecular weight (MW) markers used in this experiment were: catalase (CAT), 232,000, phosphorylase B (PHS-B), 94,000, bovine serum albumin (BSA), 67,000, ovalbumin (OVA), 43,000, and lactate dehydrogenase subunit (LDH), 36,000. LaCrosse was used as a control. Its proteins have MW as follows: lipoprotein (L) - 180,000, glycoprotein 1 ($G_1$) - 120,000, glycoprotein 2 ($G_2$) - 39,000 and nucleoprotein (N) - 25,000. Using these standards and assuming a linear relationship between MW and distance migrated, one can calculate

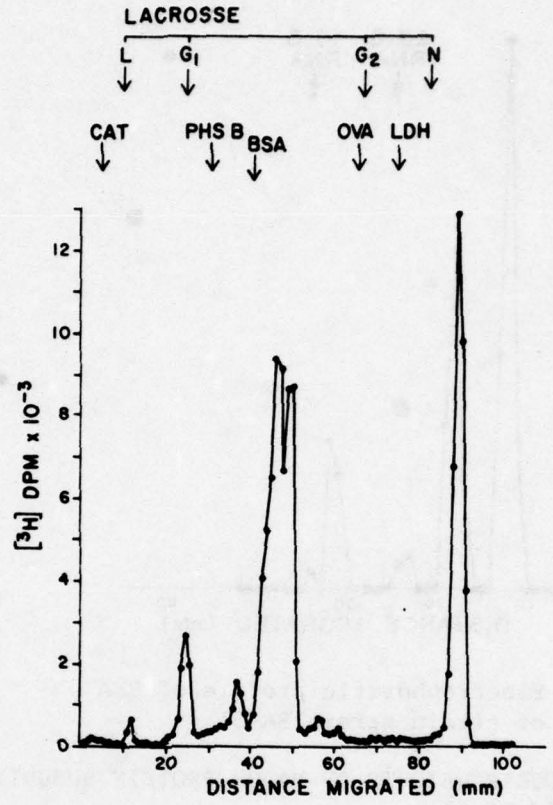


Fig. 1. Electrophoretic profile of RVF virus, strain SA-51

the MW of the various peaks in the RVF profile. The major peaks have MW of 70,000, 63,000 and 23,000, while the minor peaks are 185,000, 120,000 and 85,000. The 120,000 through the 63,000 peaks were shown to be glycoproteins by labeling the virus with glucosamine; the highest peak has been shown to be the viral nucleocapsid. The 185,000 peak corresponds with the small peak (L) of LAC which is believed by some to have transcriptase activity. The 120,000 peak was not always this prominent, in fact sometimes it was not even present. The large glycolysated peak and nucleoprotein were always present. Sometimes this spike appeared as a single peak, or as a peak with a trailing shoulder. On 12% acrylamide gels this was clearly separated into 2 peaks.

The profile shown in Figure 2 is very similar to the previous one except that it is of SA-51 RNA on 2.4% polyacrylamide gels. The RNA was extracted from purified virus with a buffer-saturated phenol technique. The standards in this experiment were 28S and 16S Escherichia coli ribosomal RNA with MW of 1,100,000 and 560,000. The 3 large peaks were determined to be 2,600,000, 1,700,000 and 600,000. The 2 small peaks were most likely BHK cell ribosomal RNA; on subsequent experiments they were not present.

Table VI summarizes the molecular data for the SA-51 isolate and compares it to values for Bunyamwera, LAC and UUK.

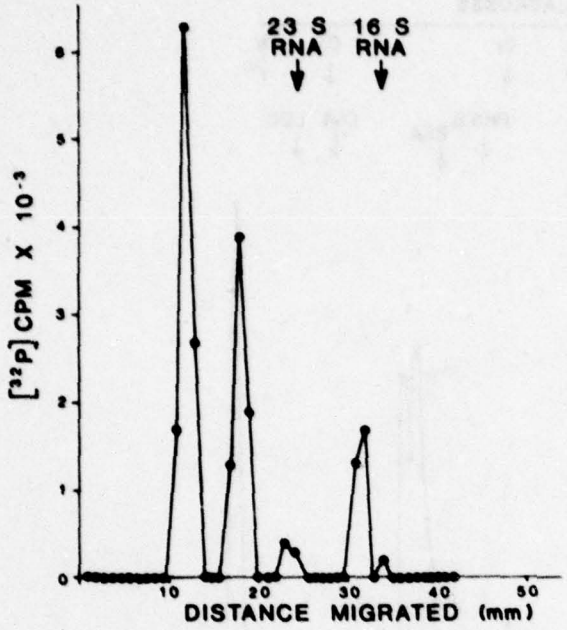


Fig. 2. Electrophoretic profile of RNA of strain strain SA-51

TABLE VI. MOLECULAR WEIGHTS OF MAJOR PROTEIN SUBUNITS AND RNA OF RVF-STRAIN SA-51

| | MW | | | | |
|---|---|---|---|---|---|
| | SA-51 | | Bunyamwera | LAC | UUK |
| | 8% PAGE | 12% | | | |
| Proteins | 70,000 | 61,000 | 125,000 | 120,000 | 75,000 |
| | 63,000 | 51,000 | 30,000 | 39,000 | 65,000 |
| | 23,000 | 26,000 | 22,000 | 25,000 | 25,000 |
| RNA | 2,600,000 | | 3,000,000 | 2,900,000 | 2,700,000 |
| | 1,700,000 | | 1,900,000 | 1,800,000 | 1,200,000 |
| | 600,000 | | 340,000 | 400,000 | 700,000 |

Tentative. All data have not been analyzed.

To date it can be safely said that the molecular and physical data are compatible with the assignment of RVF virus to the family Bunyaviradae. This study also generated the groundwork necessary to do comparative morphological studies of other isolates of RVF virus with emphasis on those isolates from man.

## LITERATURE CITED

1. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

## DISCUSSION

Dr. Nathanson: Does each segment of the genome code for one or several proteins or polyproteins?

Captain Rice: Evidence available from Dr. Bishop's work, primarily with California group viruses, would lead you to believe that. He has been able to develop temperature-sensitive mutants which show differences in one of the viral proteins, which is the fingerprint of one of the genome segments.

Dr. Nathanson: Were there more than 3 proteins?

Captain Rice: The minor proteins in RVF virus are somewhat different from Bunyamwera viruses. They are similar to the phlebotomus fever viruses that Dr. Bishop has looked at.

Dr. Nathanson: Is there a virion polymerase?

Captain Rice: I am not sure; some people say the L protein has some transcriptional activity. I don't know for certain.

# RIFT VALLEY FEVER VACCINE PRODUCTION

Francis E. Cole, Jr.
Virology Division

Formalin-killed Rift Valley fever (RVF) vaccine has been in use since the early 1960s. This vaccine was prepared from the Entebbe strain of RVF virus grown in primary African green monkey kidney cells (1, 2).

Currently there are only about 45,000 doses of the old product left. In view of the potential need for larger quantities of this product as indicated in Major Peters' presentation, the Salk Institute (formerly Merrell-National Drug Co.), under contract to the U.S. Army Medical R & D Command was tasked in March 1978 with producing 150,000 doses of RVF vaccine.

The virus used as seed material by Salk was the same Entebbe strain, but one which had been passed 2 times in pretested diploid fetal rhesus lung (FRhL) cells (3). This virus seed was re-certified by current standards, that is, shown to be free of adventitious agents, bacteriologically sterile, etc. (4).

Due to the nonavailability of "clean" primary green monkey kidney cells, the new RVF vaccine was prepared in the diploid FRhL cells, which were developed for vaccine use by the Bureau of Biologics. They have been used by the WRAIR to produce a live, attenuated dengue-2 vaccine that has been tested recently in volunteers (5). These cells are bank-frozen; they have been rigorously tested as to sterility, absence of an adventitious agents, lack of oncogenicity and for normal diploid karyology.

Production methods are illustrated very succinctly in Figure 1. As indicated, FRhL cell cultures were prepared in a separate area, washed and

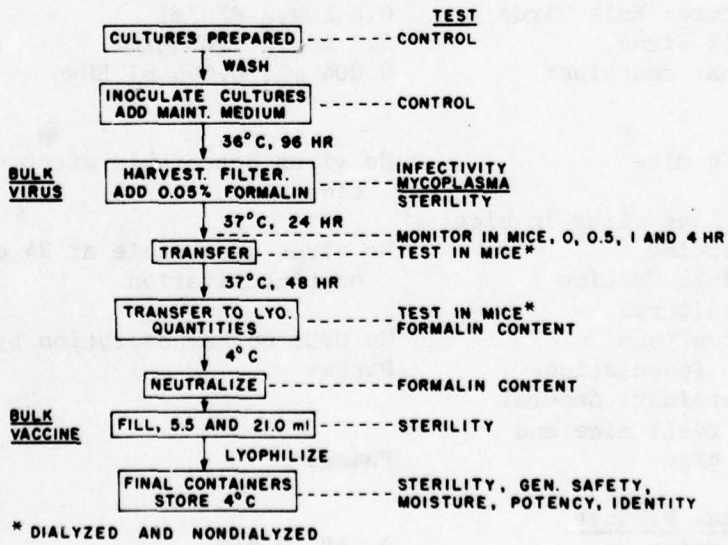


Fig. 1. RVF vaccine production scheme

sent into a "hot" laboratory suite where they were inoculated with seed virus. Virus was harvested after incubation of the cultures at 36 C for 96 hr. The pooled harvest, after filtration, (Bulk Virus) was sampled for subsequent testing as shown on the right.

Formalin for inactivation was added to the Bulk Virus to a final concentration of 0.05%; the process was carried out at 37 C with continuous stirring. An inactivation curve was determined by titration of samples taken at 0, 0.5, 1 and 4 hr after addition of formalin (Monitor test in mice). The contents of the inactivation vessel were transferred at 24 hr to a sterile vessel by siphoning and the inactivation continued for 48 hr. Samples were taken at 24 and 72 hr after formalin addition to permit testing in mice for residual live virus.

At the end of the 72-hr inactivation period the lot was split into quantities suitable for lyophilization of 5- and 20-dose vials; after short-term holding at 4 C this Bulk Vaccine was sampled for sterility testing and dispensed for lyophilization. The final containers (i.e., final lyophilized product) were tested as shown.

Table I is a summary of the basic tests conducted on Lot 1, runs 1 and 2; the results are typical of those seen with the 6 lots produced and

TABLE I. RVF VACCINE--SUMMARY OF TESTING (LOT 1, 2 RUNS)

| Test | Results |
|---|---|
| Sterility: Bulk Virus | Passes (incl. Mycoplasma + tuberculosis) |
| Bulk Vaccine | " |
| Final container | " |
| Control fluid | " |
| **Potency** | |
| 1. Cell culture: Bulk Virus | 6.6 $Log_{10}$ PFU/ml |
| 2. Mice: Bulk Virus | 5.3 $Log_{10}$ $IPLD_{50}$/ml |
| 3. Mice: final container | 0.004 ml, 0.005 ml $ED_{50}$ |
| **Safety** | |
| 1. Monitor in mice | No virus detectable after 4 hr inactivation |
| 2. Residual live virus in mice: | |
| Bulk Vaccine | No virus detectable at 24 or 72 hr postinactivation |
| dial. Bulk Vaccine | |
| 3. Control cultures | |
| a. Observations | No CPE; no hemadsorption by day 18 |
| b. Mouse inoculation | Passes |
| 4. Final container: General Safety test: mice and guinea pigs | Passes |
| **General - Final Product** | |
| 1. Residual moisture | 0.45%, 0.24% |
| 2. Residual formalin | 0.008%, 0.004% |

fully tested thus far. Standard sterility tests on samples taken from in-process and the final dried product were all negative. Tests for bacterial contamination (6) were conducted with all samples, while the Bulk Virus was also tested for Mycoplasma (7) as well as Mycobacterium tuberculosis using Lowenstein-Jensen medium.

Potency tests were conducted in two ways. First, the Bulk Virus pre-inactivation titers were assayed by PFU determination and mouse titrations; titers ranged from $10^{6.4}$-$10^{7.0}$ in the former and from $10^{5.3}$-$10^{6.7}$ in the latter. Second the standard potency test in mice (1) conducted with the final product gave $ED_{50}$ values similar to the vaccine now in use.

With regard to safety testing: (a) the monitor test in mice (inactivation curve) indicated a killing time of $\leq$ 4 hr; (b) no residual live virus was demonstrable in samples taken at 24-72 hr after the addition of formalin, as tested in weanling mice; (c) control cultures as well as samples of fluids from these cultures showed no evidence of adventitious agents; and (d) the standard final container tests (8) for safety in guinea pigs and mice resulted in no deaths or untoward reactions; animals showed normal weight gains for the test period. Both residual moisture (< 1.0%) and residual formalin (<0.01%) in the final container lot shown were at acceptable levels in each case.

To get a better perspective of the time-frame involved in producing multiple large-scale lots of a killed product one need only follow Lot 1 through the various stages of production as seen in Table II. Although the cells are already prepared, virus is inoculated, harvested, inactivated and freeze-dried by the end of the 7th week, it is not until the end of the 12th and 13th weeks that Lot 1, Runs 1 and 2 are completely tested. Thereafter, barring unforseen circumstances, a new lot would be completed every 2 weeks. However, due to difficulties with the lyophilizer at the Salk Institute, Lots 5 and 6 were not completed until November 1978, rather than the projected completion time of September 1978. Therefore, considering a lead time of $\approx$2 months the 150,000 doses required slightly more than 7 months to produce and fully test.

The magnitude of this production effort is great. For a period of $\approx$6 months there was a total commitment of the physical plant to the task, including 4 biological containment suites totaling more than 12,000 $ft^2$ with 36 laboratory rooms. In addition 4500 $ft^2$ of the facility were used in direct support of the production effort, viz., glassware preparation, normal animal holding rooms, cage washing area, and freeze-dry area, where the product was filled, dried and labeled.

At the outset the staff at the Salk facility was organized into teams led by professionals with expertise in cell culture (1 PhD + 4 technicians), vaccine production (1 PhD, 1 BS + 5 technicians) and quality control (1 PhD + 2 technicians). Many of the skilled technicians had sufficient cross-training to permit transfer of team members to other areas based on temporal requirements of the production effort.

The use of this team approach is believed to have held production costs to a minimum. The estimated cost of the 150,000 doses thus far produced should be between $5.00 and $6.00 a dose. These figures are based

TABLE II. PRODUCTION SCHEDULE FOR 6 LARGE 25,000-DOSE LOTS OF RVF VACCINE

| Month | Final Container Tests | Test for Residual Live Virus | Week[a] | Cell Preparation | Virus Inoc. | Virus Inactivated | Freeze-dried Vaccine |
|---|---|---|---|---|---|---|---|
| April | | | 1 | LOT 1 | | | |
| | | | 2 | LOT 2 | LOT 1 | | |
| | | | 3 | LOT 3 | LOT 2 | LOT 1 | |
| | | | 4 | LOT 4 | LOT 3 | LOT 2 | |
| | | LOT 1 | 5 | LOT 5 | LOT 4 | LOT 3 | |
| | | LOT 2 | 6 | LOT 6 | LOT 5 | LOT 4 | LOT 1 - RUN 1 |
| | | LOT 3 | 7 | | LOT 6 | LOT 5 | LOT 1 - RUN 2 |
| | | LOT 4 | 8 | | | LOT 6 | LOT 2 - RUN 1 |
| | | LOT 5 | 9 | | | | LOT 2 - RUN 2 |
| | | LOT 6 | 10 | | | | LOT 3 - RUN 1 |
| | | | 11 | | | | LOT 3 - RUN 2 |
| | LOT 1 - RUN 1 | | 12 | | | | LOT 4 - RUN 1 |
| | LOT 1 - RUN 2 | | 13 | | | | LOT 4 - RUN 2 |
| | LOT 2 - RUN 1 | | 14 | | | | LOT 5 - RUN 1 |
| | LOT 2 - RUN 2 | | 15 | | | | LOT 5 - RUN 2 |
| | LOT 3 - RUN 1 | | 16 | | | | LOT 6 - RUN 1 |
| | LOT 3 - RUN 2 | | 17 | | | | LOT 6 - RUN 2 |
| | LOT 4 - RUN 1 | | 18 | | | | |
| | LOT 4 - RUN 2 | | 19 | | Lead-time: ≈2 months | | |
| | LOT 5 - RUN 1 | | 20 | | Total production time for | | |
| | LOT 5 - RUN 2 | | 21 | | 150,000 doses: ≈7+ months | | |
| | LOT 6 - RUN 1 | | 22 | | | | |
| Sept. | LOT 6 - RUN 2 | | 23 | | | | |

[a]Tests are completed at week shown

on a total commitment time of between 6 to 7 months on a contract that costs about $757,000/6 months.

It is important to realize that the Salk Institute facility is the only commercial-scale laboratory in the country which has both the proper, approved facilities and a fully trained and experienced staff capable of producing the RVF vaccine. The Army is indeed fortunate to have such a staff and laboratory at its disposal.

In closing it should be noted that data from the first 3 lots of vaccine have been forwarded by the Salk Institute to USAMRIID. Currently an application for clinical use of this product is being prepared by our staff. Upon completion it will be submitted to the Army Investigational Drug Review Board for approval for use in man.

## LITERATURE CITED


1. Randall, R., C. J. Gibbs, Jr., C. G. Aulisio, L. N. Binn, and V. R. Harrison. 1962. The development of a formalin-killed Rift Valley fever virus vaccine for use in man. J. Immunol. 89:660-671.

2. Randall, R., L. N. Binn, and V. R. Harrison. 1964. Immunization against Rift Valley fever virus. Studies on the immunogenicity of lyophilized formalin-activiated vaccine. J. Immunol. 93:293-299.

3. Wallace, R. E., P. J. Vasington, J. C. Petricciani, H. E. Hopps, D. E. Lorez, and Z. K. Kadanka. 1973. Development and characterization of cell lines from subhuman primates. In Vitro 8:333-341.

4. Code of Federal Regulations. April 1, 1977. Title 21, Part 600. U. S. Government Printing Office, Washington, DC.

5. Eckels, K. H., W. E. Brandt, V. R. Harrison, J. M. McCown, and P. K. Russell. 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. 14:1221-1227.

6. Code of Federal Regulations. April 1, 1977. Title 21, Part 610.12. U. S. Government Printing Office, Washington, DC.

7. Code of Federal Regulations. April 1, 1977. Title 21, Part 610.30. U. S. Government Printing Office, Washington, DC.

8. Code of Federal Regulations. April 1, 1977. Title 21, Part 610.11. U. S. Government Printing Office, Washington, DC.


## DISCUSSION

Dr. Benenson: Would you review for me once again the facilities outside the U. S. for making this vaccine?

Dr. Cole: Other than South Africa, there is a hint of availability from Israel.

Colonel Barquist: South Africa and Israel are both in some state of planning, but to our knowledge, are not producing Rift Valley fever vaccine.

Dr. Elisberg: Just to alert you, if this product ever gets to the point to be considered for licensing, I don't think there have been adequate safeguards or quality control testing. As a review, consider what was required for the inactivated poliovirus vaccine. If the Rift Valley fever product had been modeled after the polio program, the Bureau of Biologics would be less concerned. This is a general statement and I have not furnished any details; what becomes important is the number of doses safety-tested in mice. For any inactivated product, the total amount of doses tested for safety is critical. Again using the polio program as a model, 4,500 human doses were safety-tested for each lot. That is not practical here but conceptually this is what will be required. I wonder about filtration after inactivation, whether or not this would have been a wise procedure. With formalin inactivation, you have the potential for clumping, and lack of particles being exposed to the formalin. Also, there was no test of the virus bulk previous to neutralization. If you are concerned about extraneous agents you have an available assay system; for example, we require a spectrum of cell cultures inoculated with the virus bulk which has been sufficiently neutralized with antisera to prevent growth of the RVF virus.

Dr. Cole: We have to cut a fine line between polio vaccine to be used in the general public and an experimental vaccine with limited use. We have to bend just a little, not to the point where anyone of us will be afraid to take the product or for that matter, give it to our own family. I think there has to be a compromise between the polio product and some of the killed products which were approved initially but have since been taken off the market. The safety test can not use up all of the product produced. As you know, its hard to get a review of any protocol from the Bureau of Biologics, a commitment review, so we do the best we can. The risk:benefit ratio must be considered; I believe we far exceed it.

Colonel Barquist: The question of filtration was looked at, was it not? Wasn't there a log of virus lost?

Dr. Cole: They filtered previous products without a large loss in titer; this was done on these earlier products because they contained a lot of junk. They were very crude, and there was a good chance that the formalin would not reach all of the virus. However, this particular RVF product is clean; it has been through filtration just prior to the addition of formalin. There is no precipitate formed. The product is crystal-clear and there have been no problems with residual live virus.

Colonel Barquist: I hope Dr. Elisberg that we have misled you with the simplicity of our presentation rather than insufficient work on our part. We will explore this further.

Dr. Elisberg: No, I don't want to leave the audience with the impression that you have not done your work. I have not seen the details and

it is very important to see total numbers. I just wanted, at this stage of the proceedings, to interject a note of my concern, concern based on what I heard. I suggest after I have seen the details, perhaps this will not be a problem.

Colonel Barquist: We plan to continue this effort with another 6 lots as scheduled.

Dr. Nathanson: You talked about doses. How is a dose defined?

Dr. Cole: We are using the dose and schedule that was established years ago; 1 ml is given on 0, 10 and 28 days; at one year, a booster of 1 ml is administered. Dr. Meadors is currently studying the dose schedule. We might not need this much antigen.

Dr. Nathanson: Then 150,000 ml would be considered 150,000 doses? Potency and dose have not necessarily been quantitated and related?

Dr. Cole: That is correct. We hope that an effective dose would be 0.5 ml and administered only twice.

Dr. Nathanson: One of the Salk vaccine problems of the Cutter Laboratory experience was lot size; the larger the lot, the better it was from the standpoint of safety testing. Large lots reduce the expense of testing. Within this reference, why only 25,000 doses of RVF vaccine per lot?

Dr. Cole: The limiting factor is freeze-drying capacity and limited storage facilities for the bulk vaccine prior to drying. Storage time should be minimized. It so happens that 25,000 doses is the ideal marriage, i.e., 2 freeze-drying runs for one lot of bulk material equals 25,000 doses.

Dr. Pappenheimer: I suggest combining lots and freezing just one large lot.

Dr. Elisberg: We must define what is considered a lot. It is possible to prepare a large bulk lot provided it has uniform properties, has been mixed together in a common vessel and all safety tests are derived from the mixture. The bulk lot can be subdivided, frozen into small quantities, and processed further into individual final fills.

Colonel Barquist: We have discussed the need for greater scale-up, i.e., roller bottles and greater freeze-drying capability. But in this particular situation, since a new cell substrate was used, the best approach seemed to be with 6 lots, reducing the probability of failure if they were combined into a single pool.

# TREATMENT OF ARENAVIRUS AND TOGAVIRUS INFECTIONS WITH RIBAVIRIN

Edward L. Stephen, MAJ, VC
Animal Assessment Division

We have previously described the successful treatment of influenza and Sendai virus infections of mice and influenza infection of squirrel monkeys using the antiviral drug, ribavirin (Virazole, 1- -D-ribofuranosyl-1,2,4-triazole-3-carboxamide). This compound was developed and patented by International Chemical and Nuclear Corp. The company has accumulated sufficient data to approach the end of phase 2 of Food and Drug Administration approval. Certain preclinical studies have recently been approved. The major effort being undertaken at the present time is a large double-blind study involving approximately 1,000 patients in 5 medical centers. This study will characterize the effect of ribavirin against naturally occurring influenza.

Since ribavirin is a broad-spectrum antiviral drug with significant antiviral activity against a variety of RNA and DNA viruses, we sought to characterize its activity against several viruses of interest to the military. The model viruses used are shown in Table I. Most of the research effort has centered around members of the toga-, arena-, and bunyaviruses groups. In order to expand our understanding of the activity of ribavirin as expeditiously as possible, we have collaborated with a number of investigators. The studies with Chikungunya (CHIK), Pichinde (PIC) and Lassa fever (LAS) viruses have been done with Dr. Jahrling (Virology Division). Studies with Japanese encephalitis (JE) virus were done by Major Harrington (Animal Assessment Division); studies with dengue virus (DEN) were done in collaboration with Colonel Russell and his group at WRAIR and Dr. Cole (Virology Division). The studies with Machupo (MAC) and Junin (JUN) viruses were done with Colonel Eddy and Rift Valley fever (RVF) studies with Major Peters (Virology Division).

The *in vitro* data so far accumulated with ribavirin are also shown in Table I. The least sensitive virus was RVF, with VEE and sandfly fever (SFS) viruses also being relatively resistant.

The *in vivo* data serve as introduction to specific comments which follow. If one were primarily interested in treating togavirus infections, the lack of correlation between *in vitro* activity and *in vivo* activity in mice, might discourage one from further testing. This lack of correlation can perhaps be explained by the fact that ribavirin does not achieve high concentrations in the brain. We have only completed the evaluation of ribavirin against yellow fever (YF) in the primate model. In those experiments, 2 of 4 monkeys treated initially 8 hr after virus inoculation survived, whereas, YF infection in rhesus monkeys is uniformly lethal. Studies are in progess with CHIK and DEN-2. Data in arenavirus models appear to be uniform through *in vitro* experiments and experiments in rodents and monkeys in that all systems consitently show positive antiviral activity.

We have also been able to demonstrate antiviral activity in RVF-infected mice and plan to do a similar study in infected monkeys. Monkeys develop viremia with no marked overt clinical symptomatology.

TABLE I. ANTIVIRAL ACTIVITY OF RIBAVIRIN IN VARIOUS TEST SYSTEMS

| Virus Group | Identification[a] | In vitro | Rodents | Monkeys |
|---|---|---|---|---|
| Toga- | VEE | +/- | -[b] | ND[c] |
| | CHIK | + | NM[b] | ND[c] |
| | JE | + | - | ND |
| | YF | + | - | +/- |
| | DEN-1 | + | NM | NM |
| | DEN-2 | ND | NM | ND[c] |
| Arena- | PIC | + | + MHA hamsters<br>+ Guinea pigs | NM |
| | MAC | + | + Guinea pigs | + |
| | JUN | + (XJ CL-3) | ND | ND[c] |
| | LAS | + | ND | + |
| Bunya- | RVF | - | + | ND[c] |
| | SFS | +/- | NM | NM[c] |
| Myxo- | Influenza | ND | + | + |

[a]CHIK = Chikungunya; JE = Japanese encephalitis; YF = yellow fever; DEN = dengue; PIC = Pichinde; MAC = Machupo; RVF = Rift Valley fever; SFS = Sandfly fever (Sicilian)
[b]No model
[c]Studies in progress or planned

I would like to briefly describe some representative experiments with RVF in mice, PIC in MHA hamsters, and MAC and LAS in monkeys.

Figure 1 shows the percentage of survivors of RVF (200 PFU) SC infected mice treated with increasing doses of ribavirin. Untreated virus control mice began to die by day 1 or 2 with a mean time-to-death of 4-5 days;

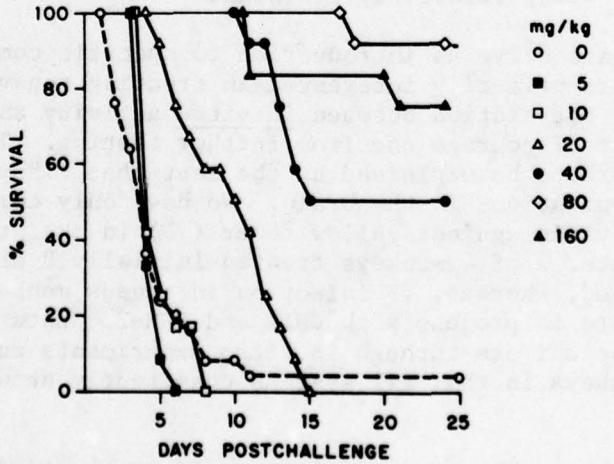


Fig. 1. Effect of ribavirin give IP to RVF-infected mice

20 mg/kg of ribavirin extended the time-to-death, but did not increase total survival. When the dose was 40 mg/kg or higher, survival was significantly increased. Figure 2 shows the effect of oral ribavirin given in the drinking water on RVF infection in mice. Early deaths were prevented. The principal target organ in RVF-infected mice is the liver, whereas, deaths in the orally treated mice were from an encephalitic syndrome. The mechanism of the encephalitis has not been determined.

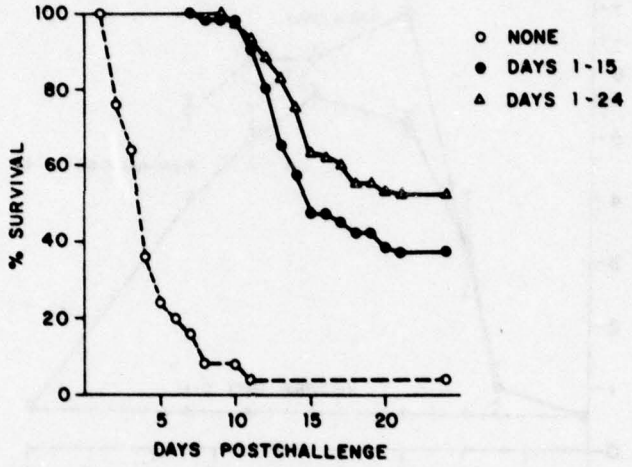


Fig. 2. Effect of oral ribavirin on RVF-infected mice

When hamsters are challenged SC ($10^4$ PFU) with a lethal variant of PIC, the mean time-to-death in untreated animals is 10 days (Fig. 3).

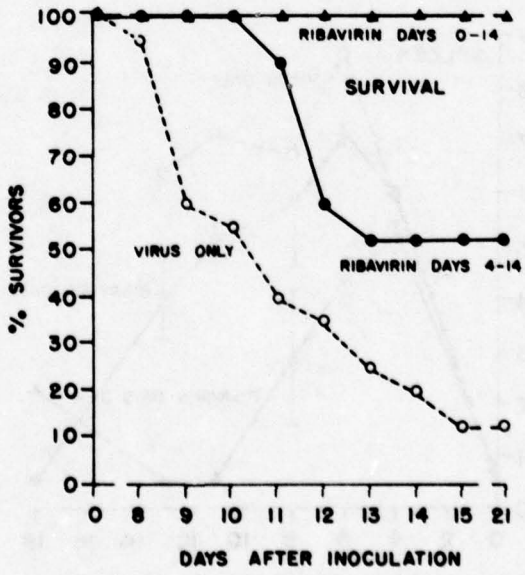


Fig. 3. Effect of ribavirin on PIC-infected MHA hamsters

Ribavirin given on days 4-14 delayed the time-to-death and increased survival; hamsters treated on days 0-14 did not die. Figure 4 shows the effect of treatment on viremia. Untreated hamsters had detectable viremia by day 5 reaching a peak by day 6. Treatment on days 4-14 attenuated the rise in viremia by day 6. Hamsters treated on days 0-14 were not

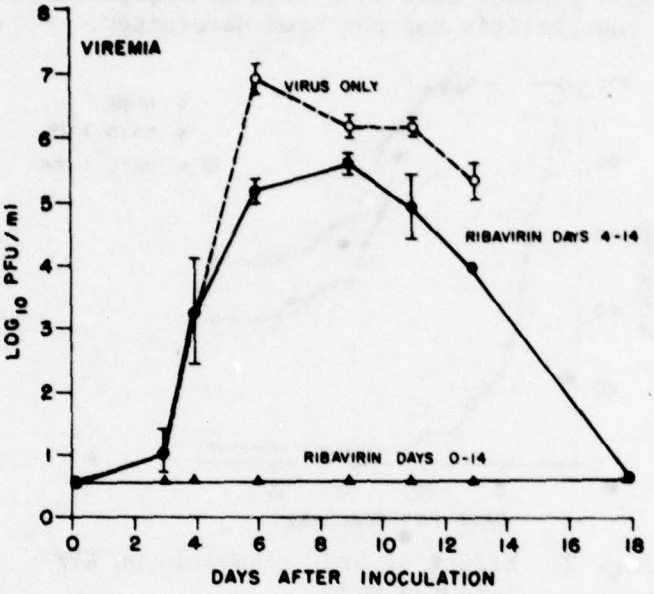


Fig. 4. Effect of ribavirin treatment on viremia

detectably viremic. The importance of extending therapy sufficiently long is demonstrated for spleen in Figure 5, in which it can be seen that spleen concentrations were quite high on days 4, 6 and 8.

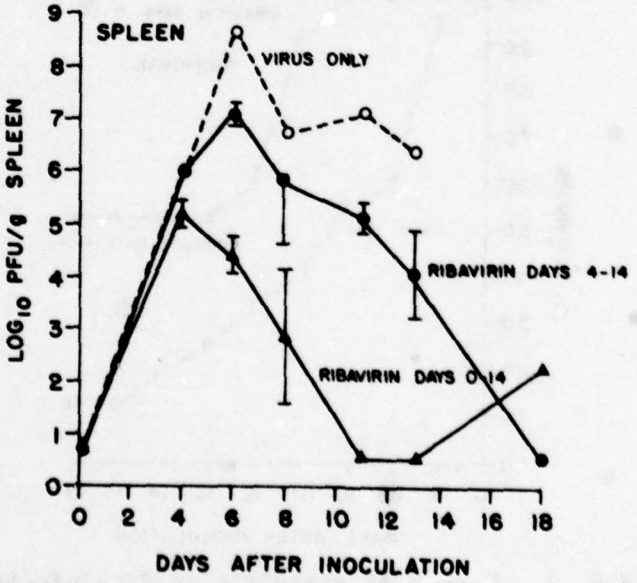


Fig. 5. Presence of virus in spleen

Preliminary in vitro data using MAC-infected Vero cell cultures are shown in Table II. In a standard yield reduction assay, MAC grows to about $10^6$ PFU/ml on days 3-6. Ribavirin (32 μg/ml) significantly reduced the yield of virus on all days. Having shown that ribavirin was active against PIC in hamsters and MAC in cell culture, we evaluated ribavirin in the well-characterized monkey model of BHF as shown in Figure 6.

TABLE II. IN VITRO ACTIVITY OF RIBAVIRIN AGAINST MAC VIRUS IN VERO CELL CULTURE

| | $Log_{10}$ PFU/ml by amount of ribavirin (μg/ml) | | | |
|---|---|---|---|---|
| Day | 0 | 3.2 | 10 | 32 |
| 3 | 5.88 | 5.43 | 4.27 | 1.17 |
| 4 | 6.17 | 5.59 | 4.48 | 0.87 |
| 5 | 6.00 | 5.55 | 5.08 | 1.30 |
| 6 | 5.81 | 5.57 | 5.52 | 1.92 |
| 7 | 5.10 | 4.95 | 5.09 | < 2.0 |

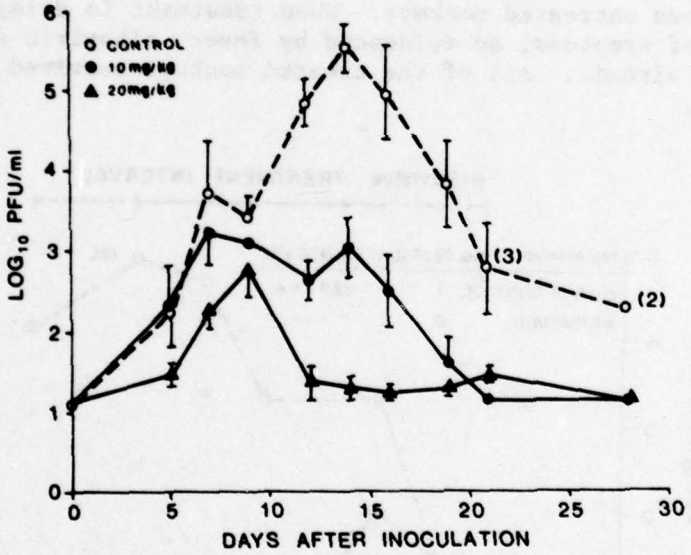


Fig. 6. Effect of ribavirin on viremia in MAC-infected rhesus monkeys

Virus control monkeys were detectably viremic by day 5 peaking by day 14. Monkeys usually died during the acute phase while still viremic. Ribavirin was given twice daily so that monkeys given 10 mg/kg/injection actually received 20 mg/kg/day and monkeys given 20 mg/kg received 40 mg/kg per day. Ribavirin did not reduce viremia until day 11. Monkeys given the higher dose had an attenuated increase in viremia and significantly lower viremia than either untreated monkeys or monkeys given the lower dose. mg/kg.

Untreated monkeys died during the acute hemorrhagic phase with a mean time-to-death of about 26 days (Table III). All ribavirin-treated monkeys surviving the acute phase, usually died during the late neurological syndrome. Having shown a strong potential for early treatment

TABLE III. CLINICAL OBSERVATIONS OF MAC-INFECTED RHESUS MONKEYS (N = 4) TREATED WITH RIBAVIRIN TRIACETATE

| Ribavirin Triacetate (mg/kg) | No. Surviving | | Mean Day to Death (+ SE) |
|---|---|---|---|
| | Initial Illness | Late Encephalitis | |
| None | 0 | 0 | 25.7 ± 3.3 |
| 10 | 4 | 1 | 71.0 ± 16.5 |
| 20 | 4 | 0 | 62.5 ± 15.0 |

using ribavirin, we next evaluated the therapeutic potential. The results of this experiment are shown in Figure 7. Again, viremia is plotted against time after virus inoculation. The virus control monkeys responded as did the previous untreated monkeys. When treatment is delayed until after the onset of symptoms, as evidenced by fever, ribavirin still had a marked effect on viremia. All of the treated monkeys survived the acute phase of illness.

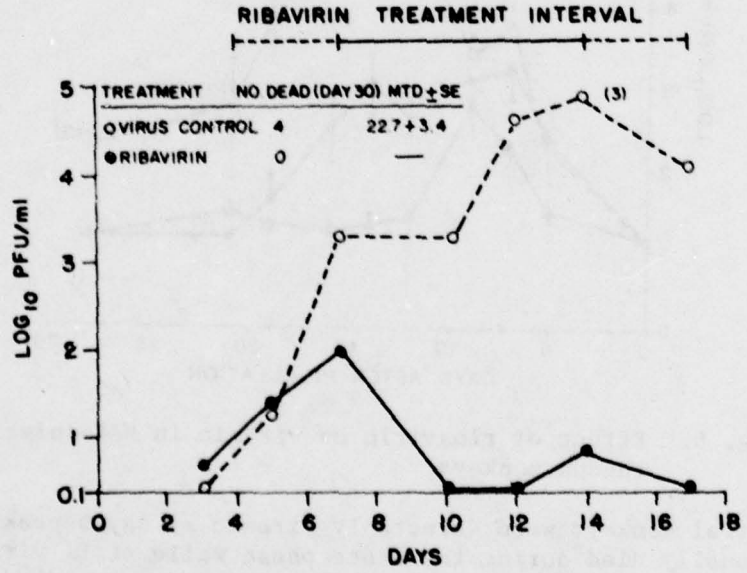


Fig. 7. Viremia titers (n=4) in ribavirin-treated rhesus monkeys

Figure 8 was prepared by Colonel Eddy and is presented to review the clinical course of MAC infection in rhesus monkeys. The acute hemorrhagic

syndrome is closely related to the viremia response and is similar to the natural disease in man. The late neurological syndrome is not a part of

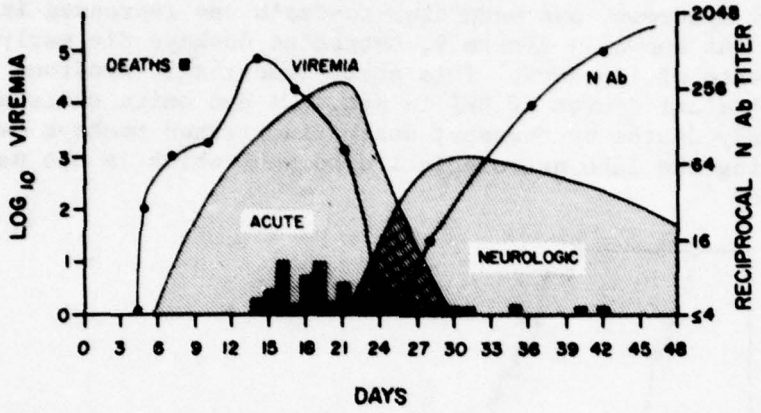


Fig. 8. Viremia, acute disease, antibody response, neurologic disease and deaths of monkeys with BHF

the disease in man. Table IV summarizes 3 monkey experiments. The third study was an experiment to confirm the therapeutic effect of ribavirin. The drug was given as before except that drug treatment was initiated on day 6 in all monkeys. I'd like to review the dosage schedules at this

TABLE IV. EFFECT OF TREATMENT ON SURVIVAL AND TIME-TO-DEATH OF MAC-VIRUS-INFECTED MONKEYS

| Study | Group | % Survival | | MTD |
|---|---|---|---|---|
| | | Day 35 | Day 90 | (Days ± SE) |
| 1 | Saline | 0 | 0 | 26 ± 3 |
| | Ribavirin triacetate | | | |
| | 10 mg/kg/injection | 100 | 25 | 71 ± 17[a] |
| | 20 mg/kg/injection | 100 | 0 | 63 ± 15[a] |
| 2 | Saline | 0 | 0 | 23 ± 4 |
| | Ribavirin | 100 | 25 | 43 ± 6 |
| 3 | Saline | 25 | 0 | 36 ± 21 |
| | Ribavirin | 100 | 25 | 39 ± 2[a] |

[a]Sacrificed when paralyzed

time since they were varied. In the first experiment ribavirin was given twice daily at 0800 and 1600 hours beginning on day 0 and continuing through day 17. The total daily dose was then either 20 or 40 mg/kg. In the second study, a loading dose concept was used, followed by subsequent doses at 8-hr intervals for 10 days. In the third experiment, the monkeys were given a loading dose of 25 mg/kg followed by subsequent injections of 5.0 mg/kg for 10 days beginning on day 6. It is apparent

that the dose of ribavirin, the treatment schedule and the time of initial treatment were not the factors limiting success since all of the treated monkeys survived beyond day 35. We were not able to prevent the late neurological syndrome, but mean time-to-death was increased in treated monkeys. As shown in Figure 9, untreated monkeys die early with a mean time-to-death of ≈20 days. This acute hemorrhagic syndrome correlates with the clinical course of BHF in man. We can quite successfully prevent these early deaths by therapy; deaths in treated monkeys begin after day 35 during the late neurological syndrome, which is not part of the human disease.

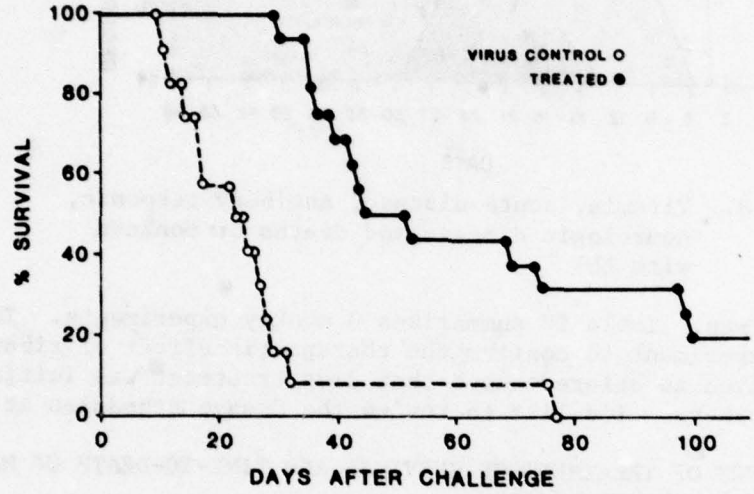


Fig. 9 Cumulated survival of MAC-infected monkeys treated with ribavirin

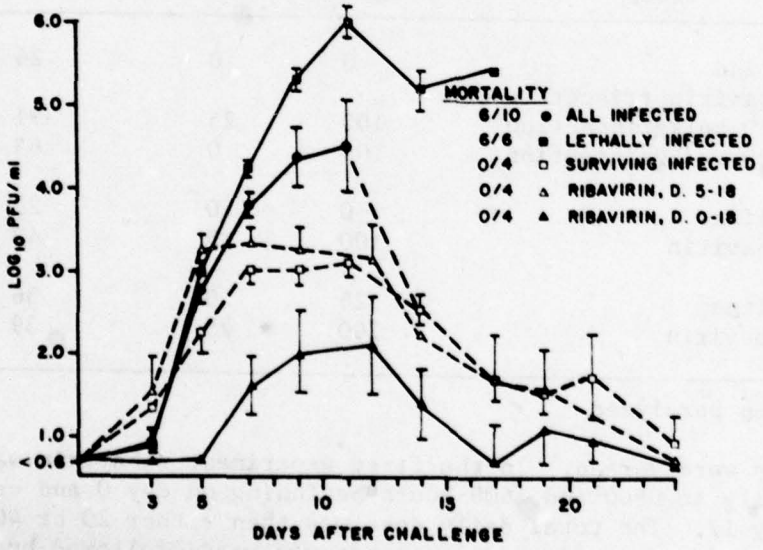


Fig. 10. Viremia in LAS-infected rhesus monkeys

The Lassa fever data presented in Figure 10 show viremia as a function of time after virus inoculation of rhesus monkeys. Viremia of all

injected monkeys (solid circles) was first detectable on day 3 and reached a peak by day 11; 6 of 10 infected, untreated monkeys died.

We have separated the viremia response of lethally infected monkeys (solid squares) from surviving monkeys (hollow squares). Monkeys that die have significantly higher viremias compared to monkeys that survive.

When ribavirin is given from days 5-18, all treated monkeys survive and their viremia response (hollow triangles) resembles that of the surviving monkeys in the virus control group suggesting that late treatment with ribavirin assists in preventing the rise in viremia >4.0 logs, thus, contributing to survival in treated monkeys. Monkeys treated from days 0-18 had a delay in onset and significantly lower viremia (solid triangles). All of these monkeys survived.

Certain studies being conducted by others are worth noting to complete the picture. The group at Porton, England, is going to evaluate ribavirin in Ebola virus-infected monkeys. There are plans to treat JUN virus infection clinically in South America and Dr. Karl Johnson is planning studies to treat LAS in Sierra Leone.

## DISCUSSION

Dr. Jordan: How does this drug work?

Major Stephen: It is thought to work by inhibiting enzymes specifically related to virus multiplication. The specific enzymes in question are not well defined, but it is known that the drug works intracellularly and inhibits enzymes responsible for virus replication. Its toxicity is well defined; doses of >10 gm are tolerated in man without severe reactions. Occasionally anemia begins on day 5 or 6. The drug is teratogenic in rats and hamsters but not in baboons.

Dr. Pappenheimer: What happens to antibody production in ribavirin-treated animals?

Major Stephen: In one study, a Machupo study using 10-20 mg of drug/kg, antibody was detected in the treated monkeys; it is not, in untreated monkeys because they generally die before antibody develops. Monkeys treated with 10 mg/kg developed earlier and higher levels of antibody than those given 20 mg/kg. I don't know why.

Dr. Pappenheimer: It seems that they should have made antibody before they died, unless they were making virus so rapidly.

Colonel Eddy: Neutralizing antibody does not appear until about day 25-28, and that seems to be a characteristic of many arenavirus diseases. Antibody does not appear as promptly as one would expect.

Dr. Pappenheimer: Why did the virus titer drop after several days?

Colonel Eddy: Because of antiviral effects of the drug.

Dr. Pappenheimer: Yes, but treatment started on day 0, titers did not drop several days thereafter.

Major Stephen: This is perhaps due to a complex relationship between virus doses and half-life of the drug. The drug half-life in rats is 8 hr but it has not been established yet for monkeys. In the first experiment, the drug was administered at 8 AM and 4 PM, then 8 AM the next day; the latter time lapse is too long and the drug is probably completely metabolized. The drug level falls below the minimal inhibiting concentration and the virus is able to multiply between the 4 PM and 8 AM period.

Dr. Benenson: We have a Machupo monkey model that survives the acute phase of disease but is then followed by an encephalitic death later on. As I recall, this observation has been extended to Rift Valley fever. In monkeys, this seems to be a consistent observation. This has not been observed in man at all; is that right?

Dr. Johnson: That is right! It does not and has not happened in man, in several hundreds of human infections.

Major Stephen: There is a difference between encephalitis in RVF and Machupo infections. Colonel Eddy has not isolated virus in late Machupo encephalitis but virus is present in Rift Valley fever encephalitis.

Dr. Benenson: You do have the data showing that late encephalitis has occurred in Rift Valley fever? How frequently in humans?

Major Peters: In humans, we don't have a good figure. I guess it is on the order of 0.1-1%.

Major Stephen: We plan to develop an RVF encephalitic mouse model to test analogs of ribavirin.

Dr. Johnson: In our clinical trial which is getting underway now in Sierra Leone, we are alert to, and concerned about, the possibility that encephalitis might occur. We have not seen any cases of late encephalitis with Lassa, based on several hundreds of cases in man. We did have 3 or 4 patients who had acute meningeal signs, similar to the related choriomeningitis due to lymphocytic choriomeningitis (LCM) virus. Please remember that Lassa and LCM share many more antigens with each other than they do with the new world arenaviruses. We were able to recover Lassa virus from spinal fluids on two occasions. I think this means that in some patients the virus does get into the brain. We will be acutely aware of, and look for, the possibility that ribavirin might be immunosuppressive and convert the disease to late encephalitis.

Dr. Benenson: May it be hypothesized that in the Machupo late encephalitis, the cause is the antigen-antibody complex, not viral infection?

Dr. Johnson: That seems to be a reasonable speculation but it has not been proven.

Major Stephen: In one experiment with ribavirin-treated monkeys infected with Lassa fever, the monkeys survived for ≈100 days with no signs of encephalitis. Two other experiments that are now 30 days old provide similar results.

Dr. Woodward: In primate studies with yellow fever, using stabilized poly(ICLC), you achieved a limited effect. Were the doses similar?

Major Stephen: These results are similar to poly(ICLC). These data suggested that an 8-hr yellow fever treatment would correspond to day 6 of Machupo infection. The dose in yellow fever was higher than in the Machupo studies. Yellow fever is quite different, because the monkeys die in 5-6 days with very high virus titers.

Dr. Johnson: It is well to remember that the strain of yellow fever you are using is no longer conventional yellow fever, not as we know it in nature. Maybe you should consider replacing this strain; perhaps obtain a strain more closely related to those strains that exist in the jungle. The Asibi strain is highly adapted to the monkey and kills animals before any immune response can be initiated.

# TOXIN RECEPTORS ON HOST CELLS

John L. Middlebrook
Pathology Division

Infection by Pseudomonas aeruginosa is a frequent complication for immunologically impaired individuals. Of particular concern to the Army is an estimate that 60-70% of the mortality encountered with burn patients, surviving the initial trauma, involves Pseudomonas sepsis. My conversation with an Israeli physician who administered to casualties in the 6-Day War convinced me that this sepsis is a serious problem indeed. While the pathogenesis of Pseudomonas infection is complicated, there is now good evidence to believe that a lethal exotoxin, termed exotoxin A, is a contributing factor. Thus it was decided to embark upon a project designed to elucidate the mechanism of action of Pseudomonas exotoxin (PE) with the ultimate aim of designing therapeutic countermeasures. For reasons which should become self-evident, we also spent some time studying a toxin related to Pseudomonas toxin, namely diphtheria toxin (DE). Our initial successes occurred with the latter; it is mostly those results which will be discussed. However, our efforts with PE have not gone entirely unrewarded. I have some preliminary data with that system as well. I would like to emphasize that this project has been a joint venture with Dr. Stephen Leppla and Ms. Rebecca Dorland.

Diphtheria toxin is a single polypeptide of 60,000 MW. As produced in culture a significant amount of the toxin is cleaved in the region of a disulfide loop by endogenous protease. This cleavage can also be effected by treatment with trypsin. In either case, cleavage results in the production of 2 fragments, termed A and B. Fragment A is an enzyme which covalently links the ADP ribosylating portion of NAD to an intracellular enzyme called elongation factor 2 (EF-2). Fragment B has no known biological activity, but apparently is required for the toxin to recognize its cellular receptor. Thus it is generally believed that DE initially binds to a specific receptor at the cell surface via the B fragment. Thereafter, by a very important but poorly understood process, at least fragment A is internalized. The fate of fragment B is unclear. Once inside the cell, fragment A expresses its enzymatic activity, carrying out the ribosylation of EF-2. Ribosylated EF-2 is inactive at its normal cellular function in protein synthesis and the cell dies.

The situation with PE-fragment A is not nearly as well understood. PE is also a single polypeptide of 66,000 MW. Whether or not there is specific cleavage of the polypeptide chain is unclear. There is some evidence for the involvement of specific fragments. However, one undisputed point is that PE has an ADP-ribosylating activity apparently identical to that of DE. The reasons for studying both toxins thus became obvious.

Early in the course of our studies we screened a large number of mammalian cell lines for their sensitivities to both PE and DE (1). Three appeared to be most sensitive, Vero, BS-C-1 and MK-2. We assumed that these cell lines might have more receptors and chose to use them for binding studies.

Fig. 1 shows the binding of diphtheria toxin to Vero cells; the difference between the labeled and excess unlabeled toxin is referred to as specific binding; it is quite clear that the degree of specificity is high. The error bars show the SE which, when not shown, were smaller than the size of the symbol. The kinetics of binding at 37 C show an increase for 1-2 hr, followed by a decrease to a steady state at a level about half the maximum binding. We refer to this pattern as biphasic. The molecular events responsible for this kinetic pattern are not entirely clear but similar biphasic binding kinetics have been observed in several polypeptide hormone systems. The kinetics of binding at 4 C (right) are also shown. At this temperature toxin does not go into the cell; interpretation of binding data is more straightforward. Here again the specificity is high and the data are reproducible.

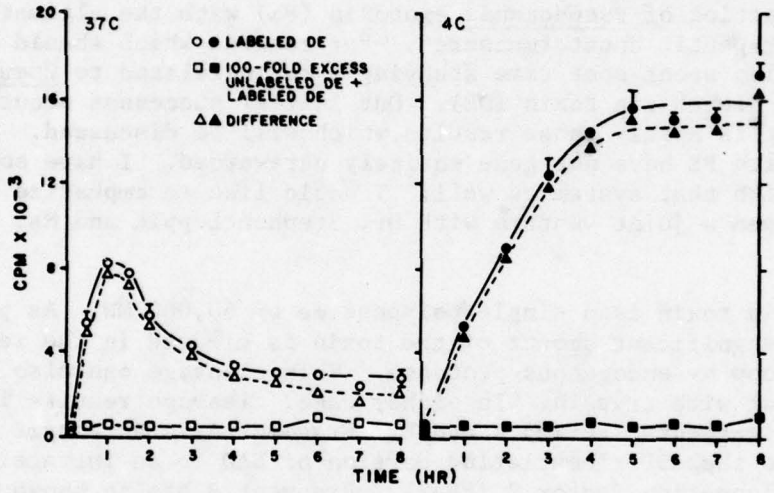


Fig. 1. Kinetics of $^{125}$I-diphtheria toxin association with Vero cells

At 4 C it is possible to make certain biophysical measurements of the binding. Fig. 2 shows a binding isotherm demonstrating that the binding is saturable. When the specific binding is plotted by the method of Scatchard, as shown in the inset, it is possible to estimate the number of binding sites/cell (B) at 100,000-200,000 and the binding affinity(B/F) ranging between 0.5-1 x $10^9$ L/mol.

Finally, it is possible to demonstrate reversibility of binding by a classical chase experiment (Fig. 3). Cells are prebound with labeled toxin at 4 C. At 0 time a chase of excess unlabeled toxin is added and the level of toxin bound to cells is measured as usual. Clearly, disassociation takes place. We calculate an off-rate constant of approximately 2 x $10^3$ $min^{-1}$.

Thus binding in this system satisfies the criteria which have come to be accepted for the demonstration of a drug or a hormone binding to its receptor: specificity, high affinity, saturability and reversibility.

We believe it insufficient to simply demonstrate specific binding and call it a receptor. Rather, some efforts should be made to determine whether

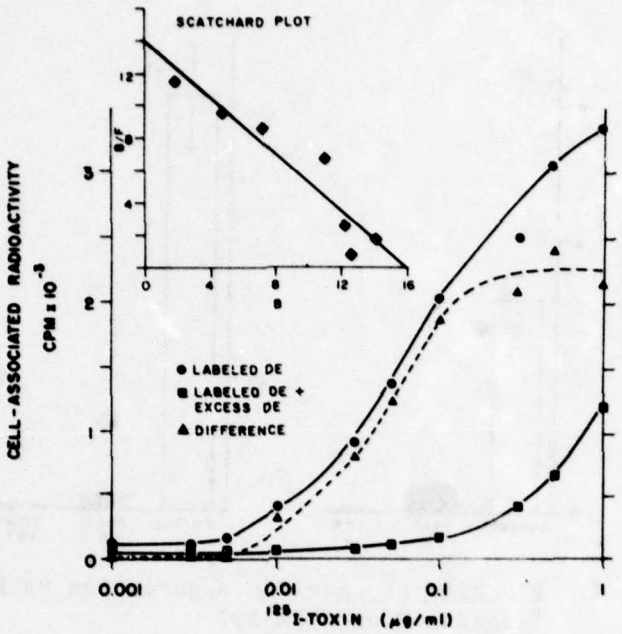


Fig. 2. Binding isotherm

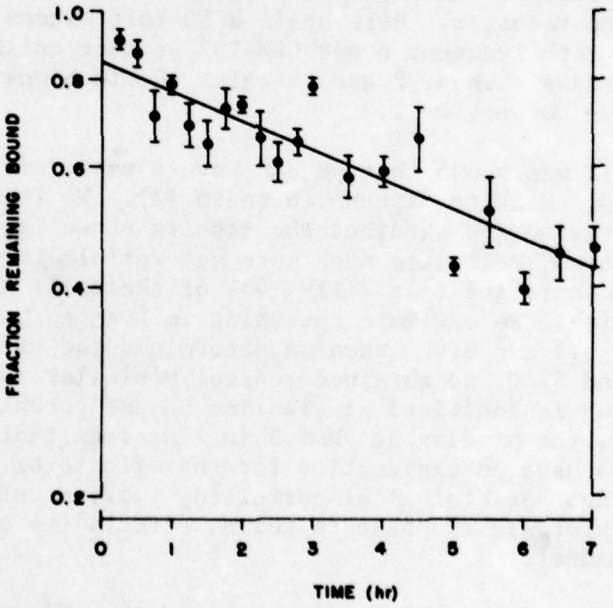


Fig. 3. Toxin-cell dissociation at 4 C

the binding one measures is related to the biological response. To this end several biological correlates have been developed to our specific binding. In this case we examined the abilities of fragment B or a toxin homologue, CRM-197, to compete for binding (Fig. 4). Fragment B is the portion

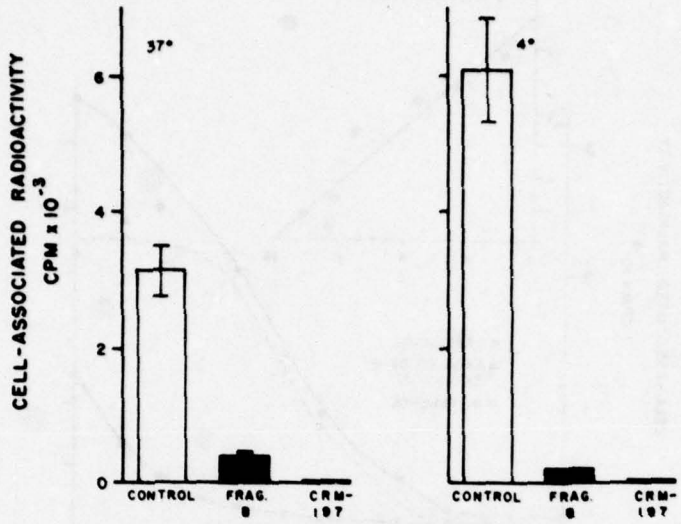


Fig. 4. Blocking of specific association by DE, fragment B and CRM-197

of DE toxin which mediates binding to the receptor. One would predict that fragment B would compete for the binding of labeled toxin; obviously a 50-fold excess does so quite well at both 4 and 37 C. CRM-197 is a mutant form of the toxin lacking a functional A fragment, but which is fully capable of binding to the receptor. Here again a 50-fold excess of CRM-197 is a good competitor. Both fragment B and CRM-197 protect cells from DE when they are added in excess. Thus, 2 agents which should block the binding of DE to its receptor do so very well.

Some time ago it was shown that pH can have a major effect on the cytotoxic response of cells to diphtheria toxin (2). We reinvestigated this effect in our system and obtained the results shown in Fig. 5. First, as shown by the squares, cells are much more susceptible to DE at acid than alkaline pH. A concentration that kills >90% of the cells at acid pH, kills <10% at alkaline. We estimate the shift in $LD_{50}$ to be about 50- to 100-fold between pH 5.5 and 9.0. When we determined the effects of pH on binding at both 4 and 37 C, we obtained generally similar patterns demonstrating that binding is inhibited at alkaline pH and potentiated at acid pH. Quantitatively, the binding at pH 5.5 is 100 times that at pH 9.0. Thus, not only do we have an explanation for the effects of pH on cytotoxicity, we also have another biological correlate, i.e., a culture condition which affects the cytotoxic response to toxin, affects the binding measured in the same manner.

In another series of experiments we compared the effects of exogenous nucleotides on binding and cytotoxicity. In this particular experiment, we examined the adenosine nucleotide series (Table I). If we look first at the left column which is the effects of nucleotides on DE-induced cytotoxicity, a clear pattern is seen. The longer the phosphate chain the better protector the compound is. Thus adenosine tetraphosphate is about twice as potent as adenosine diphosphate while adenosine monophosphate barely

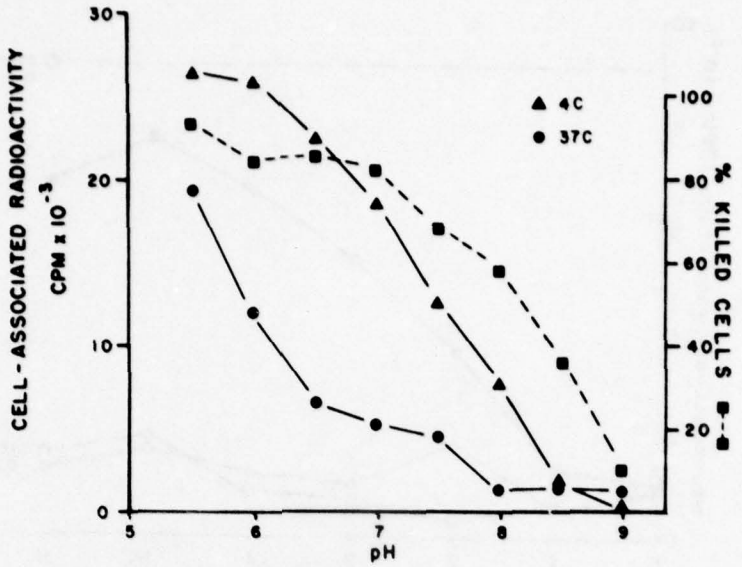


Fig. 5. Effect of pH on $^{125}$I-DE cell association and cytotoxicity

shows activity. Tripolyphosphate, the phosphate oligomer portion of ATP shows some protection but much less than the intact nucleotide. If we now look at the effects on specific binding we see that both down and across the line, similar patterns are observed. This correlate, plus the others we have developed, place us in the position to state that the specific binding measured involves the receptor which mediates the biological, i.e., cytotoxic, response of cells to DE toxin (3).

TABLE 1. EXOGENOUS NUCLEOTIDE EFFECTS ON RADIOLABELED DE-CELL ASSOCIATION

| Nucleotide | mM required to: | |
|---|---|---|
| | Block 50% of specific label-cell association | Protect 50% of the cells |
| Adenosine 5'-tetraphosphate | 0.02 | 0.03 |
| Adenosine 5'-triphosphate | 0.1 | 0.05 |
| Adenosine 5'-diphosphate | 0.25 | 0.5 |
| Adenosine 5'-monophosphate | 2 | >3 |

Having established the validity of the binding system, we are engaged in several rather obvious avenues of research. We have found, for example, that if the DE receptor is suddenly and almost completely depleted, the cell responds by rapidly placing new receptor into the membrane (Fig. 6). In this experiment we preincubated the cells with well above the saturating level of unlabeled CRM-197, which binds normally to the toxin receptor. The preincubation then serves to tie up continually virtually all the receptor with unlabeled ligand. At 0 time the unbound CRM was washed away and the cells were incubated at 37 C with fresh medium. At the times indicated, the cells were rapidly cooled and the level of new cell membrane receptor

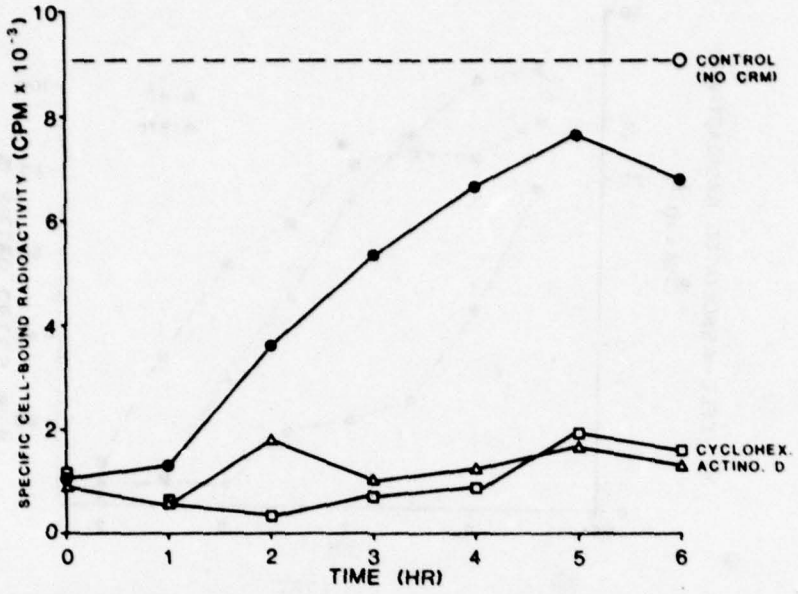


Fig. 6. Regeneration of cell-surface receptor by DE

measured by binding with radiolabeled DE at 4 C. At 0 time, very little cell surface receptor (solid circles) was available for binding. However, it is clear that the cell was able to regain available receptor quite rapidly; by 6 hr the number of cell surface toxin receptors returned essentially to control levels. The ability of the cell to regain the receptors is dependent both on protein synthesis, as shown by the effect of cycloheximide on RNA synthesis as demonstrated by the actinomycin D block. The straightforward interpretation of the data is that receptors are taken inside the cell; we are measuring the synthesis and subsequent membrane insertion of new receptor. An alternate explanation is that receptor is taken inside the cell and recycled but that its reinsertion into the membrane is dependent on protein and RNA synthesis. In either case, it appears that the cell has an optimum or desired level of cell surface toxin receptors and strives to maintain that level when it is perturbed.

Although the efforts just described have produced some interesting data, the main emphasis of research has been directed to the question of how DE is internalized in the cell in a biologically active form. This question takes on additional meaning with the recent demonstrations that many polypeptide hormones, including insulin, prolactin, parathyroid hormone, growth hormone, gonadotropins and polypeptide growth factors apparently also enter cells. It should be remembered that viruses, or at a minimum their nucleic acids, must also enter cells in a biologically functional form. Thus insights gained into the mechanism by which DE toxin enters the cell should be of widespread interest in cell biology. This very point was made quite well sometime back by Boquet and Pappenheimer (4).

Our basic attack of the problem has been 2-pronged. In one approach, which we term the biochemical one, we use radiolabeled toxin and follow the course of its internalization and processing by the cell. By the application of various drugs or physiological conditions, we have been able to

infer something of the events and subcellular systems involved in DE internalization. We first developed an assay which permits the differentiation of cell-surface bound and internalized toxin. What is done is that after washing to remove noncell-bound toxin, cells are treated with protease. That radioactivity which is chewed-off and released, we believe, represents toxin bound at the cell surface. That which is not susceptible to protease action is internalized. In the example shown in Fig. 7, prebound cells at 4 C were washed and either kept at 4 C or raised to 37 C at 0 time; almost all the radioactivity can be released. As time increases at 37 C, the protease-releasable counts decrease considerably while the protease-resistant counts increase until the 2 values are about equal. This pattern presumably reflects internalization of the toxin, since at 4 C, where internalization does not occur, there is little or no change in either protease-releasable or -resistant counts. Thus, we have an assay which can distinguish between toxin at the cell surface and that which has been internalized.

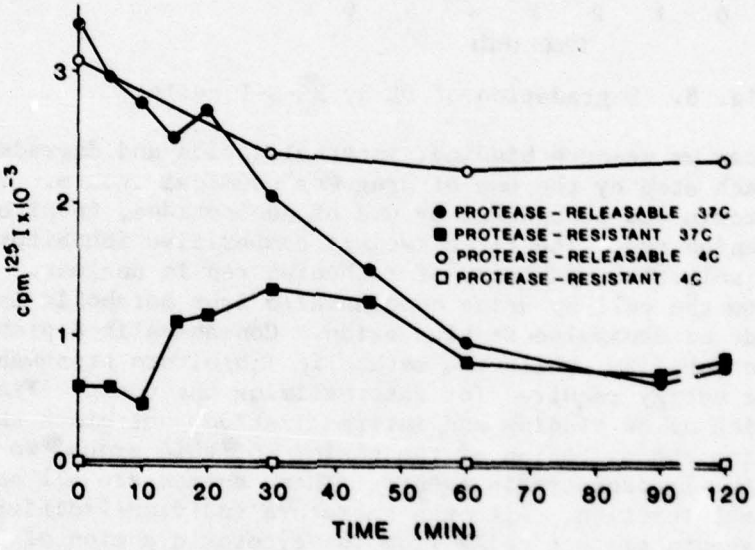


Fig. 7. Internalization of DE by Vero cells

A second assay developed was designed to measure the degradation and excretion of toxin by cells. This assay involves first washing the cells to remove unbound toxin, then sampling the medium above the cells at various times for the release of trichloroacetic acid (TCA)-soluble radioactivity. The TCA-soluble radioactivity is of course a measure of degraded and excreted toxin. An example of this assay is shown in Fig. 8. Cells were prebound at 4 C, washed at 0 time and either raised to 37 C or left at 4 C. At the lower temperature, no toxin goes inside, and as expected the total cell counts remain approximately constant; TCA-soluble radioactivity decreases drastically. Concomitantly TCA-soluble radioactivity appears in the media. Thus we have available assays which can determine the total cell-bound radioactivity, that fraction which is on the surface, that which is internalized, and finally the fraction which has been degraded and released.

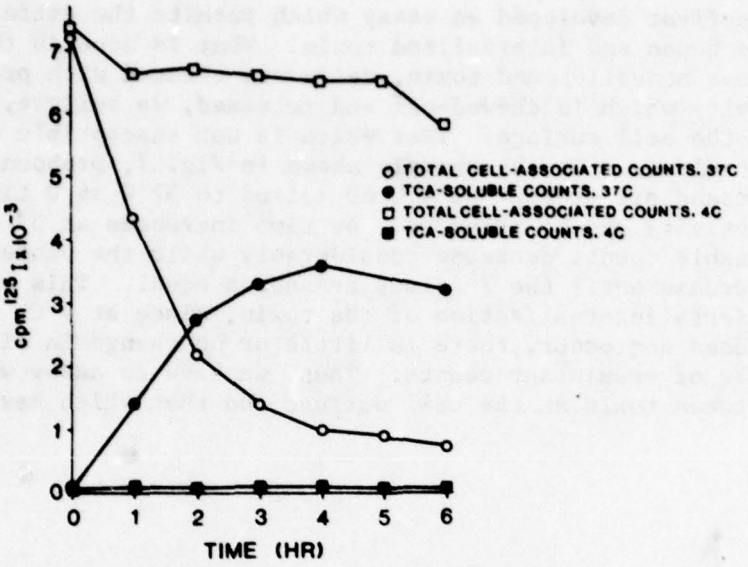


Fig. 8. Degradation of DE by BS-C-1 cells

Not only can we measure binding, internalization and degradation, but we can block each step by the use of drugs or chemical agents. Initial binding of DE toxin can be blocked by use of nucleotides, inositol polyanions or ruthenium red. The first two are competitive inhibitors of binding, while the molecular mechanism of ruthenium red is unclear. We can block entry into the cell by using concanavalin A or metabolic inhibitors, such as fluoride or deoxyglucose plus azide. Concanavalin A probably inhibits membrane mobility, while the metabolic inhibitors presumably deprive the cell of the energy required for internalizing the toxin. Finally, we have agents which allow binding and internalization, but block the subsequent degradation and excretion of the toxin. Of this group, we are most interested in the lysosomotropic agents. These agents are all believed to inhibit lysosomal function. Although there are individual differences, as a class these agents protect cells from the cytotoxic action of DE and inhibit the release of TCA-soluble radioactive fragments. These results led us to the conclusion that lysosomal processing is a necessary step for the intoxication of cells by the receptor-mediated mechanism. These then are some of the results of our biochemical approach to the problem of toxin internalization.

In the other approach, which we term morphological, we enlisted the aid of Dr. John White and the electron microscope to visualize where the toxin might be located at various points in the internalization process. In this approach, efforts with DE have been unsuccessful, but those with PE have been encouraging.

During the past 2-3 years, an elegant model for the internalization of some macromolecules has been worked out, primarily by Goldstein and Brown (5) of the University of Texas. Briefly, the model holds that receptors for certain macromolecules are localized in easily identifiable structures

on the cell membrane which are known as "coated pits." Coated pits regularly invaginate, pinch off and enter the cell as coated vesicles. The vesicles ultimately fuse with lysosomes and processing takes place. When we used the immunochemical staining procedure of Sternberger et al. (6), we found that PE also binds to coated pits. Binding with toxin was carried out at 4 C so that it would all be at the cell surface. Invagination occurred forming a deep pit. Thus, we believe that at least PE enters cells by a coated-pit transport mechanism.

In summary, we developed a radiolabel-based binding system for DE which is well suited to elucidating the mechanism by which this toxin enters the cell in a biologically active form. We attained limited but important information about PE as well. We believe insights gained by the study of these toxins should be of interest to researchers studying the action of polypeptide hormones as well as those interested in toxins. Furthermore, we believe it more than coincidental that the lysosomotropic agents protect from DE but are also inhibitory to the replication of several viruses. An electron micrograph obtained by Simpson and colleagues (7), showed that vesicular stomatitis virus is taken up into cells by coated pits. While we are reasonably certain that all viruses are not taken up by them, it may be that some of those of interest to USAMRIID are, and our findings might be an aid in this area as well.

## LITERATURE CITED

1. Middlebrook, J. L., and R. B. Dorland. 1977. Response of cultured mammalian cells to the exotoxins of Pseudomonas aeruginosa and Corynebacterium diphtheriae: differential cytoxicity. Can. J. Microbiol. 23:183-189.

2. Duncan, J. L., and N. B. Groman. 1969. Activity of diphtheria toxin. II. Early events in the intoxication of HeLa cells. J. Bacteriol. 98:963-969.

3. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with Vero cells. Demonstration of a receptor. J. Biol. Chem. 253:7325-7330.

4. Boquet, P., and A. M. Pappenheimer, Jr. 1976. Interaction of diphtheria toxin with mammalian cell membranes. J. Biol. Chem. 251: 5770-5778.

5. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. Annu. Rev. Biochem. 46:897-930.

6. Sternberger, L. A., P. H. Hardy, Jr., J. J. Cuculis, and H. G. Meyer. 1970. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. 18:315-333.

7. Simpson, R. W., R. E. Hauser and S. Dales. 1969. Viropexis of vesicular stomatitis virus by L cells. Virology 37:285-290.

DISCUSSION

Dr. Benenson: Since you are basing the bulk of your data on radioactivity, is the radioactvity on the A or B fragment? These questions have me puzzled; is it the whole toxin molecule? Where is the radioactive label?

Dr. Middlebrook: We use a choromine-T labeling procedure, $^{125}I$ is the label and is distributed about equally between fragments A and B. This is essentially the same thing Dr. Pappenheimer found in his earlier studies.

Dr. Benenson: Does this mean that the whole molecule enters the cell?

Dr. Johnson: I would doubt that you are able to discriminate between fragments. If A and B molecules are present in equal numbers and have about equal labels on them, you are not able to say; "One is in, or both are in." I doubt it even with the reproducibility of your data.

Dr. Middlebrook: There is only one way to do it, to label preferentially the A and B fragments and we have that in mind.

Major A. Anderson: These label studies would not be able to tell the difference between degradation and destruction of one fragment and active retention of the other fragment without subsequent degradation. Since you have shown that these are coated pits, all of the material, even the material which supposedly stays in association with the membrane, is internalized and degrades. It, therefore, no longer becomes an important issue whether B stays on the membrane and allows A to enter; that is because the whole membrane is internalized.

Dr. Middlebrook: I wish that were true. The point is the coated pit. It has been shown very well with Pseudomonas data; however, we have not shown that diphtheria toxin is bound to coated pits.

# BOTULISM IMMUNE PLASMA

George E. Lewis, Jr., MAJ, VC
Pathology Division

The 7 goals of the Botulism Microbial Toxin group are: 1) develop and equip a containment facility for investigation of microbial toxins; 2) train a technical and professional staff; 3) develop methods for production of botulinal neurotoxins, types A-G; 4) develop purification methods for isolation of pure neurotoxin; 5) develop suitable toxoid methods for production of a laboratory-size lot of polyvalent botulinum toxoid; 6) obtain a stock of Botulism Immune Plasma (Human); and 7) develop in vitro assay methods for both toxin and antibody. Considerable progress has been made under the leadership of Colonel Metzger toward the attainment of many of these goals. However, it is progress toward attainment of goal number 6 that will be discussed. That is, the collection, processing, and stockpiling of Botulism Immune Plasma of human origin.

But first, I will refresh your memory as to the relevance of botulinal neurotoxins to the USAMRIID mission of conducting studies on the prophylaxis and treatment of diseases initiated by biological agents.

Figure 1 presents the prediction curve of effectiveness of aerosol dissemination. The efficiency of botulinal neurotoxin type "A" as a formidable BW agent is attested to by the 50% lethality prediction at 10 km downwind. Other plausible threats provided by the use of botulinal neuro-

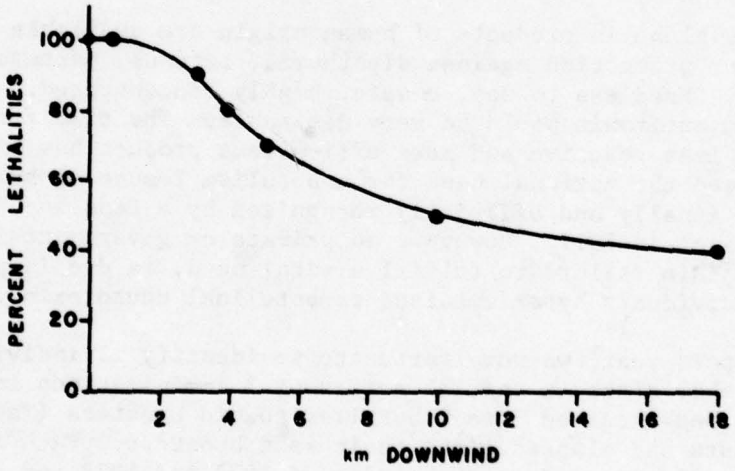


Fig. 1. % lethality with downwind distance after release of $10^{6.3}$ $LD_{50}$ of agent relative to meters as a ground level line source

toxins are highlighted in 3 newspaper accounts during the past year.

The first clipping reflected a recent incidence of suspected botulism in Clovis, NM, in which 29, type A neurointoxicated individuals were hospitalized. An Air Force Sergeant was the index case for this as yet unre-

solved outbreak. The next clipping from Atlanta, GA, entitled, "Water Poison Threat Revealed," was a reminder of the practicality of botulinal neurotoxins as "tools for terrorists."

The final Washington Post clipping, "Risk of Botulism Seen In Feeding Honey to Infants Under 1 Year," brought to light a recently described syndrome, on which considerable national attention is now focused, that of the occurrence of >100 cases of infant botulism during the past year and a half, and their relationship to the sudden infant death, or crib death, syndrome.

Botulism, unlike many of the conditions studied at USAMRIID, is a disease resulting from an intoxication rather than an infectious process. Infant and wound botulism are 2 notable exceptions. Specific treatment of botulism is directed primarily toward the neutralization of circulating botulinal neurotoxin by the administration of botulinum antitoxin and supportive care, such as the maintenance of respiratory function; however, CDC consistently reports a 21% rate of adverse reactions to the antitoxins used.

At this time last year, which was 81 years after the first suggested use of botulism antitoxin, and many anaphylactic reactions later, the national supply of botulism antitoxin consisted solely of 4 heterologous products, 2 from Connaught Medical Research Laboratories, Botulism Antitoxin, Type E (Equine) and Botulism Antitoxin Trivalent (Equine), Types A, B and E; Serum Mod Botulismus, Type A, B, C, D, E og F (Hesteserum) (Statens Seruminstitut, Copenhagen) and Bivalent Botulism Antitoxin (Equine Origin), Types A and B (Lederle).

Immune serum globulin products of human origin are available and routinely used for protection against diphtheria, tetanus, pertussis, rabies, and zoster. Needless to say, a safe, highly concentrated, human-derived, botulism antitoxin would be very desirable. The time for the development of a less reactive and more efficacious product has long been upon us, and indeed the national need for a Botulism Immune Globulin of human origin was finally and officially recognized by a Food and Drug Administration panel in 1977. However, no private or governmental agency saw fit to act. This failure to fulfill a vital need, is due in part, to the rarity of individuals hyperimmunized to botulinal neurotoxins.

Early this past year, we were fortunate to identify 11 individuals at Fort Detrick who, after an initial series of 3 immunizations in the 1950s and 1960s, had received 3 to 8 botulism toxoid boosters (Table I). However, 7-12 years had elapsed since their last boosters. Each individual was subsequently boosted in the spring of 1977 and 1978 and then recruited as a donor of Botulism Immune Plasma. Serum titers to 3 types of botulinal neurotoxin were determined before and after the 1978 booster. As anticipated from the notorious history of the toxoid currently employed, the type B titers were regretably low. But, the type A and E titers were satisfactory (Table II).

In cooperation with the blood donor center at WRAMC we have collected by plasmapheresis >70 L of human plasma from individuals hyperimmunized to

TABLE I. BOOSTER HISTORY OF BOTULISM IMMUNE PLASMA (HUMAN) DONORS

| Donor | No. boosters before 1977 | Years since last booster | Donor | No. boosters before 1977 | Years since last booster |
|---|---|---|---|---|---|
| 1 | 5 | 8 | 7 | 7 | 7 |
| 2 | 3 | 12 | 8 | 7 | 3 |
| 3 | 8 | 7 | 9 | 5 | 11 |
| 4 | 7 | 8 | 10 | 7 | 8 |
| 5 | 8 | 10 | 11 | 5 | 8 |
| 6 | 8 | 7 | | | |

TABLE II. NEUTRALIZATION TITERS OF DONORS

| Donor | Units/ml | | | | | |
|---|---|---|---|---|---|---|
| | Type A | | Type B | | Type E | |
| | Before | After | Before | After | Before | After |
| 1 | 28.9 | 129.0 | 3.2 | >6.0 | ND | >18.3 |
| 2 | ND | 6.1 | ND | 0.2 | ND | 2.3 |
| 4 | 3.2 | 5.1 | 0.8 | 0.8 | 1.9 | 2.2 |
| 5 | 4.0 | 13.0 | 0.3 | 0.5 | 1.9 | ND |
| 6 | 1.0 | 8.2 | 0.3 | 0.4 | 3.8 | 11.4 |
| 7 | 0.4 | 8.1 | ND | 0.5 | ND | 2.3 |
| 8 | 20.0 | 25.8 | ND | 0.1 | ND | 8.9 |
| 9 | 14.5 | ND | 0.3 | 0.8 | 9.2 | ND |
| 11 | 6.4 | 12.8 | ND | 1.5 | 6.6 | ND |

5 types of botulinal neurotoxins. Titers against types A, B and E are shown in Table III.

TABLE III. DONATION HISTORY OF DONORS

| Donor | Donations | | Highest titer (units/ml) | | |
|---|---|---|---|---|---|
| | No. | Liters | A | B | E |
| 1 | 12 | 6.805 | 129.0 | 6.1 | >25.6 |
| 2 | 12 | 7.660 | 6.2 | 0.3 | 2.3 |
| 3 | 2 | 1.098 | 15.4 | ND | ND |
| 4 | 14 | 8.510 | 16.3 | 0.8 | 2.2 |
| 5 | 10 | 6.840 | 13.0 | 0.5 | 1.9 |
| 6 | 12 | 6.405 | 8.2 | 0.9 | 11.4 |
| 7 | 10 | 6.335 | 8.6 | 0.5 | 11.4 |
| 8 | 12 | 7.267 | >30.7 | 0.1 | 8.9 |
| 9 | 14 | 11.045 | >20.5 | 0.8 | 9.2 |
| 10 | 2 | 1.210 | 32.2 | 4.1 | 2.4 |
| 11 | 16 | 10.130 | 18.1 | 4.8 | 6.6 |

This plasma was recently granted an IND number by the Bureau of Biologics. Most of this Botulism Immune Plasma will be custom fractionated early

This plasma was recently granted an IND number by the Bureau of Biologics. Most of this Botulism Immune Plasma will be custom fractionated early next year by the Michigan State Health Department and subsequently bottled as Botulism Immune Globulin (Human), for which an IND number will be sought.

Thus the advice "The use of humans as production animals has recently been proved to be not only effective but practical," offered over 24 years ago by Petty (1) to the First National Botulism Symposium, was finally heeded. Humans thus far are effective and practical donors of Botulism Immune Plasma. A second and much larger pool of potential human donors has since been recruited. We are currently attempting to establish a program for the collection of 1,000 L of Botulism Immune Plasma over a 2-year period.

We are unaware of any instances of hypersensitivity, as occur with heterologous horse products, resulting from the administration of immune plasma or gamma globulin of human origin. Botulism Immune Plasma (Human) and Botulism Immune Globulin (Human), will have the additional advantage of an extended effective half-life by remaining active in a patient's serum much longer than equine gamma globulin. Thus an effort to develop a Department of Defense, and the only national, stockpile of Botulism Immune Plasma and ultimately Botulism Immune Globulin of human origin is well under way.

In conjunction with the end-product oriented studies just described, we have also conducted several concurrent pilot studies of an applied research nature to demonstrate the efficacy of homologous antitoxin as compared to heterologous antitoxin in the prevention and treatment of botulism in guinea pig model systems. In a series of experiments, guinea pigs were challenged with type E neurotoxin, at various time intervals both before and after the IM administration of equated log dilutions of homologous or heterologous type E antitoxin (Table IV). Eighteen days after passive immunization with homologous or heterologous antitoxin guinea pigs were divided into 2 subgroups; half the guinea pigs were again challenged with 5.0 $GPLD_{50}$, while the second half received a 4-fold greater challenge. The survival percentages were quite similar for both homologous and heterologous antitoxins, when, toxin was given 18 hr before or 24 hr after antitoxin. But, a drastic difference in survival was recorded when pigs were rechallenged 18 days later. Similar experiments were conducted to evaluate types A and B antitoxins. Both homologous and heterologous antitoxins proved to be efficacious for the prophylaxis of types A, B and E neurointoxication and to a lesser degree for their therapy. The decay rates of equine origin botulism antitoxins, in a recent fatal human case of type A botulism, at Fort Benning are presented in Figure 2. Similar decay rates can be seen for types A, B and E immunoglobulins. The short half-life, ≈1 week, of the heterologous antitoxin in this patient is evident. A circulating antitoxin level of 0.25 U/µl is considered protective for prophylactic application. If such an individual had received a similar quantity of immune globulin of human origin, which has an anticipated minimal half-life of ≈3 weeks, the protective level would be exceeded for ≈22 weeks (dashed line), as compared to 3 weeks for horse antitoxin.

TABLE IV. EFFICACY OF HOMOLOGOUS AND HETEROLOGOUS ANTITOXIN GIVEN AT VARIOUS TIMES TO GUINEA PIGS CHALLENGED WITH 5.0 $GPLD_{50}$ OF TYPE E NEUROTOXIN

| Antitoxin | IU | % Survival | | |
|---|---|---|---|---|
| | | 18 hr before | 24 hr after | 18 days after[a] |
| Homologous | 0.50 | 75 | 100 | 100/100 |
| | 0.050 | 25 | 75 | 0/ 0 |
| | 0.005 | 0 | 50 | ND |
| Heterologous | 0.50 | 75 | 100 | 0/ 0 |
| | 0.050 | 0 | 75 | 0/ 0 |
| | 0.005 | 0 | 50 | ND |

[a] 2 doses used were 5.0 and 20 $GPLD_{50}$

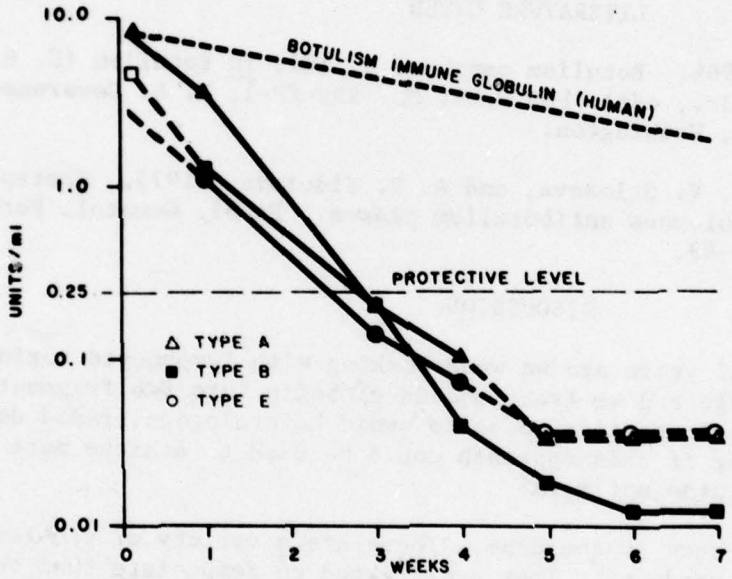


Fig. 2. Decay of trivalent botulism antitoxin (equine) in a human patient

The use of human gamma globulin in the prophylaxis and therapy of botulism would not only virtually eliminate foreign protein-initiated anaphylaxis but, unit for unit, the ratio of effectiveness and the extended half-life of human gamma globulin should be far superior to gamma globulin derived from a heterologous species.

Thus, in summary of our antitoxin studies: both homologous and heterologous antitoxins were efficacious for prophylaxis and therapy of botulism in guinea pigs; homologous antitoxins persist at and above protective levels for extended time periods; and heterologous antitoxins quickly de-

cayed to quantities insufficient to afford protection in humans and guinea pigs.

To summarize our Botulism Immune Plasma efforts: ≈70 L of Botulism Immune Plasma have been collected from Fort Detrick employees; 50 L are to be fractionated for Botulism Immune Globulin (Human) in 1979, yielding ≈166 prophylactic and as few as 83 therapeutic doses; and we are currently negotiating a contract for 1,000 L of Botulism Immune Plasma (Human) to be delivered in the spring of 1980. This 1,000 L should yield 20 L of Immune Globulin or the approximate equivalent of 3,333 prophylactic or 1,666 therapeutic doses. Lest we think, we are the only governmental group investigating the larger scale use of homologous botulism antitoxin, I wish to leave you with a portion of a recently translated Russian document dated 1975 but released only this year. "In certain instances homologous antibotulin plasma was transfused following injection of equine serum, when despite therapy with heterogenic antitoxin the patients did not show signs of improvement. The introduction of homologous antitoxin in such cases led to the elimination of intoxication" (2).

## LITERATURE CITED

1. Petty, C. S. 1964. Botulism omnibus, p. 181, In Botulism (K. H. Lewis and K. Cassel, Jr., eds). PHS Publ. No. 999-FP-1, U. S. Government Printing Office, Washington.

2. Piven, I. N., T. V. Golosova, and A. V. Sidorova. 1975. Therapeutic efficacy of homologous antibotulism plasma. Probl. Gematol. Pereliv. Krovi 20(11):46-49.

## DISCUSSION

Dr. Knight: Several years ago we were working with lymphocyte toxin for transplant usage and we fractionated globulin into Fab fragments to get rid of the Fc portion so as to avoid heterologous, rapid degradation. I wonder if this approach could be used to achieve more effective use of equine antisera?

Major Lewis: I am sure it could be. There are a variety of problems with the current equine products. They are treated to despeciate them somewhat. However the IgG is not consistently broken into Fab fragments. The feasibility of the approach could be questioned. Perhaps we could find a commercial source to fractionate the human immunoglobulin in this manner.

Dr. Pappenheimer: You are looking at a man who has had 3 ml of Lederle's Fab fragments called despeciated antibotulism anti-toxin. Years ago, I thought I had inhaled a lot of botulism toxin and had a friend inject 3 ml into me. Shortly thereafter, I had a fever of 105°, and the last thing before passing out, I told my wife, "Don't call the doctor." About one week later, I had 500 ml of blood withdrawn and tested and found that I had about 10 mg of precipitating antibody against horse globulin. I am also the first person immunized with a toxoid which I made myself. Now for my question, with regard to the

antitoxin (decay) elimination curve, why is there no evidence for immune eliminations of type A antitoxin at all? Why did it happen so fast?

Major Lewis: This individual had a type A intoxication and no difference, within the limits of the sensitivity of the test, was observed in the degradation of the 3 markers followed, A, B and E antitoxins. This patient received 4 vials of pentavalent antitoxin; there was no evidence that a substantial quantity of the A antitoxin had been bound by toxin. Elimination rates were the same for each antitoxin. This brings to mind the question: how much are we overtreating these people? We can not titrate them.

Dr. Pappenheimer: I had horse serum twice before, once with diphtheria at age 9 and tetanus when I was 6. That was something like 30 years before the inoculation already mentioned. This is in regard to your boosting. There was no question that I was well boosted by horse serum after 30 years.

Dr. Knight: I'm sure you were.

Major J. Anderson: In regard to Dr. Pappenheimer's question, by the time the patient received the antitoxin, he had essentially stabilized in neurological progression. After that, you could assume that the antitoxin was not effective. It had bound as much toxin as it was going to bind, although the patient did have circulating toxin in his blood when first tested, as Major Lewis can best describe.

Major Lewis: He had quite a substantial quantity of circulating toxin when first tested. His blood was not tested again by CDC for circulating toxin. We could found no toxin in later samples collected after administration of antitoxin.

Dr. Johnson: Please show the first slide again (Fig. 1). I have never seen anything like this before, that is, the downwind travel slide. Can anyone tell us some details as to how this was determined? Is this a theoretical model?

Mr. Larson: The estimated dose for man came from the report of the Expert Committee on Bacteriological Warfare to the United Nations. This was based on an estimated dose for man by the respiratory challenge. This dose was used in conjunction with the well-documented curves of downwind travel for particulate clouds, for specified altitudes of release, atmospheric conditions and wind speeds. Major Lewis can tell you what they are.

Major Lewis: The assumptions used were: 0.3 μg is an $LD_{50}$ for man; 40% of this was a pure product, or $1.3 \times 10^6$ $LD_{50}$/gm; disseminated 2 gm/m of line source; line length of 15 km, and 30 kg of agent required. The release altitude was ground level, wind speed, 5 mph, and atmospheric stability was moderate inversion.

Dr. Johnson: How many kilograms of toxin would have to be produced?

Major Lewis: 30 kg of crude toxin.

Dr. Johnson: Is the technology readily available to produce this quantity of toxin to satisfy all asepcts of the equation? Can the toxin be produced on a large enough scale? Can you do it in your garage?

Major Lewis: Since our job is defensive, not offensive, we constructed this equation for one narrow area to illustrate the point of toxin efficiency, using currently available data.

Colonel Metzger: A concentration of $10^{7.5}$ $LD_{50}$ represents crystalline toxin; the product used in the equation is 40% crystalline. It is very possible to produce these levels of toxin and with this degree of purity. This is what the organism literally makes sitting in the culture.

Dr. Johnson: This is the kind of answer that I expected even though I know nothing about this field. Now, my next question is: in terms of the priorities Colonel Barquist mentioned, what do you think of the feasibility of the passive antitoxin approach to a problem of this magnitude?

Colonel Barquist: You should not conclude that this is our main line defense against this agent. This is a spin-off of having to immunize people, also needing a therapeutic modality here in the United States and for any accident we may have.

Dr. Johnson: This is not a purely military problem. Lassa fever would require much more technology to become a substantial threat than would the production of this quantity of botulinum toxin. I would conclude that you have in hand a very dangerous weapon. The world is not in a mood to worry about who is in a military uniform and who is not.

Dr. Knight: When Dr. Johnson started talking, I thought he was going to say that it was far-out and improbable. I see, Dr. Johnson, that was not your point at all. I have followed these developments for many years and find that it would be possible to put together a number of potent situations; that is, stable toxic agents that can drift with the wind and cause a lot of human morbidity as well as mortality. This information is well known to people around this table. I do not think any of us can feel very comfortable, particularly in these times. As Colonel Metzger says you can almost produce this amount of toxin in the garage.

Dr. Benenson: As I understand it, antitoxin is given to neutralize the free toxin, but the toxin attached to the nerve cell or nerve plate is not affected. Perhaps the toxin wears off. Does the duration or persistence of the antitoxin make any difference in the survival of the patient?

Colonel Barquist: Today, it is not prudent, clinical judgment to administer antitoxin on suspicion, if all you have is an equine product. You may kill people with horse antitoxin. Today, you wait until you have sent your

specimen to CDC and received the answer. Antitoxin treatment then accompanies a positive answer. You are therefore ethically able to treat the patient about 72 hr later. A supply of the present product will allow us to treat the early cases on suspicion and gain the 72 hr.

Dr. Benenson: I accept greater safety; but I am interested in the pathogenesis of this case at Fort Benning. The individual had a good level of antitoxin for a long period of time. In infantile botulism babies are treated without antitoxin. Was this particular patient given artificial life support? What did he die of?

Colonel Barquist: Pneumonia, probably. He was already on an artificial respiratory machine when he got the first antitoxin.

Dr. Benenson: The argument was given that antitoxin was good for prophylaxis. To whom would you give botulism antitoxin prophylactically?

Colonel Barquist: The next 15 people after you had made the first diagnosis.

Major Lewis: It would be routinely given, in some instances to people who have been or may be exposed to the same or a similar intoxicating source.

# OTHER USAMRIID RESEARCH ACCOMPLISHMENTS

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Scientific Advisor

During the past year, the research programs of USAMRIID followed the priorities established in FY 1977 and were updated during FY 1978. These emphasize studies on some of the most virulent and pathogenic microorganisms known. Based upon agreements reached with the Center for Disease Control (CDC) and the new priorities, USAMRIID initiated studies this year with Lassa fever virus (LAS), Congo/Crimean hemorrhagic fever viruses, the bacterial agent causing Legionnaires' disease, and the anthrax bacillus. Laboratory containment suite modifications were initiated to permit the safe, simultaneous handling of all these Priority I agents as listed by Colonel Barquist, including members of the generally lethal Ebola-Marburg group of viruses.

As reviewed by previous speakers, USAMRIID and US Navy researchers became involved in an unexpected 1977-78 outbreak of Rift Valley fever (RVF) throughout Egypt, which occurred for the first time in history. Because there were thousands of cases, with the appearance of hemorrhagic disease, encephalitis, and blindness as complications, and the occurrence of many human deaths, additional emphasis was placed on in-house studies of RVF virus, diagnostic methods and viral chemotherapy. In addition, industrial production through contract was stepped up and given first priority to produce 300,000 doses of a new RVF vaccine. USAMRIID is the sole source (worldwide) of this human vaccine. Since it is quite possible that RVF could spread into other Mid-East countries, the problem has potential military medical importance.

Work was continued on other high priority organisms, including Machupo virus, the etiologic agent of Bolivian hemorrhagic fever (BHF), Korean hemorrhagic fever (KHF) virus, botulism, and dengue. These organisms all possess significant BW potential, are lethal for man and present enormous safety problems. Fortunately, USAMRIID is one of the few laboratories in the free world where such agents can be studied with minimum risk to laboratory personnel and no risk to the surrounding environment. The goal of this research is to develop safe and effective vaccines or toxoids for these highly dangerous but poorly understood diseases. Work in pathogenesis and immunogenesis continues to support these vaccine development studies.

Second-order priorities included studies on Japanese B encephalitis (JE), Argentine hemorrhagic fever, Chikungunya (CHIK), and VEE. Toxin studies continued with bacterial exotoxins and enterotoxins. New diagnostic capabilities were developed as were new treatment methods for viruses, bacteria and bacterial toxins. Rickettsial studies continued on exogenous tick-borne spotted, epidemic typhus and Q fevers. The organisms or toxins in this priority are also highly dangerous for man, possess significant BW potential and pose special problems of safety, although, at an intermediate order of magnitude. Third-order priorities included studies on western and eastern equine encephalitis (WEE and EEE), melioidosis and tularemia.

Reactivation of a dynamic new USAMRIID program in FY 1977 to permit studies in research volunteers was followed this year by extensive use of human subjects in both USAMRIID and Walter Reed Army Institute of Research (WRAIR) research studies. The new MRVS (Medical Research Volunteer Subject) projects replace the old "Project Whitecoat." Each specific project protocol must undergo extensive ethical (Human Use) and scientific committee reviews within USAMRIID and again at higher command levels before final approval is given. In other important progress regarding human studies this fiscal year, USAMRIID's clinical facilities were upgraded further for safely caring for patients with rare, highly contagious diseases and for performing diagnostic microbiologic and clinical laboratory tests on such patients. In addition, an agreement was reached with CDC to accept such potentially contagious patients in transfer from their laboratories for diagnostic studies and treatment at USAMRIID. A similar agreement has been negotiated with the Plum Island Animal Disease Center of the USDA where studies with RVF will begin in early FY 79.

Specific comments on USAMRIID progress are included under the following headings: Clinical Studies, Vaccine Development, Vaccine Adjuvant Studies, Immunological Responsiveness Studies, Pathogenesis Studies, Diagnostic Studies, Therapy and Toxin Studies.

## Clinical Studies

Clinical studies performed during FY 1978 included those on new experimental vaccines in volunteers, long-term phase III studies of the existing experimental vaccines being used to protect laboratory personnel, studies performed in collaboration with WRAIR and the National Institutes of Health (NIH), and finally studies to provide clinical care to patients admitted to the USAMRIID high-containment suite when they were suspected of having been exposed to a highly dangerous infectious microorganism.

Major areas of clinical research included 2 vaccine trials in volunteers with an inactivated Rocky Mountain spotted fever (RMSF) vaccine and the first trial in phase I testing in volunteers of a live, attenuated virus vaccine for dengue-2. The latter study was performed in collaboration with investigators from WRAIR who developed the vaccine. A major clinical study also involved the first systematic nontherapeutic evaluation in volunteers of human transfer factor; this was conducted in cooperation with research investigators from NIH.

The phase III testing of a large number of experimental vaccines was continued in laboratory workers of USAMRIID and other collaborating institutions. These vaccines were administered primarily for the safety of "at-risk" laboratory workers and included live, attenuated TC-83 VEE vaccine, inactivated EEE and WEE vaccines, inactivated phase II Q fever vaccine, attenuated live tularemia (LVS) vaccine, anthrax vaccine, inactivated RVF vaccine, inactivated CHIK accine and polyvalent botulinum toxoid. Additional assessment studies performed on both the TC-83 VEE vaccine and the killed VEE vaccine (C-84) suggested that a combination of both vaccines should be used in the future protection of laboratory workers. Initial VEE vaccination will be performed with the live TC-83 product; booster C-84 inoculations will be given to those individuals whose anti-VEE titer either

does not reach, or falls below, values deemed to be protective. This procedure will improve the safety and efficacy of the vaccination procedure.

Important new safety measures were taken during FY 78 to upgrade the abilities of USAMRIID to handle highly contagious patients. These efforts included the acquisition of additional containment equipment designed to treat a hospitalized patient within a specially constructed, plastic isolator module containing its own independent air supply and filtration system. Orders were placed to acquire additional modules for hospitalized patients and also to acquire modules designed for the transport of contagious patients within aircraft or ambulances.

To further the safety practices of USAMRIID, facilities were set aside and a training program established to permit clinical laboratory and microbiologic samples to be handled and assayed under the strictest forms of containment using pressurized plastic suits with filtered air supply. In addition, a building modification program was initiated to upgrade the unique ward facilities to permit the care of patients by hospital personnel who will be dressed in similar protective suits.

Vaccine Development

The development of new vaccines constitutes a major requirement in the USAMRIID research mission; the major aspect of this program dealt with attempts to create new vaccines against important virus diseases, with emphasis on arenaviruses that cause highly lethal hemorrhagic fevers. The viral vaccine program also included new studies on RVF, continued work to create an attenuated dengue-1 vaccine, and further research to improve vaccines for the alphaviruses, including VEE, EEE, WEE, and CHIK.

Attempts to develop a potent inactivated BHF vaccine in a certifiable substrate continued to be frustrated by low yields of virus antigen and difficulties in obtaining consistent virus inactivation. Alternative approaches to existing virus inactivation procedures were studied through contracts in an effort to resolve this problem. On the other hand, in an exciting breakthrough, the attenuated strain of Junin virus (virulent strains of which cause Argentine hemorrhagic fever) was found to protect monkeys and laboratory rodents against both the Argentine and Bolivian forms of hemorrhagic fever. Since the attenuated Junin virus has already been used in 600 human recipients in Argentina, additional studies with this attenuated virus vaccine are planned.

Another approach has been to examine the immunizing potential of subunit polypeptides obtained from the arenaviruses. Both glycoprotein and nucleoprotein subunits of Pichinde virus and the lethal Machupo virus were obtained through fractionation and purification. When used as vaccines, these purified subunits protected animals against homologous virus challenge. Similar studies were then initiated with RVF virus; again, appropriate growth, purification, concentration, and radiolabeling techniques allowed for the identification of 3 major subunits, 2 of which were glycoproteins. These will now be tested for immunogenicity.

Studies with selected clones of dengue-1 virus were continued and additional tests developed to identify virulence "markers" to permit the

selection of an avirulent virus subpopulation that could be used as a possible human vaccine. A major breakthrough in this regard was the development of a new method to detect low-titer viremia in monkeys. The absence of viremia in subhuman primates may eventually turn out to be the optimal "marker" available for selecting a vaccine candidate appropriate for human use.

In another fundamental breakthough with respect to the development of safe vaccines, it was found that hydroxylapatite column chromatography could be used to separate physically alphaviruses on the basis of differences in virion surface charge. This permitted large numbers of virus strains of VEE, EEE and WEE to be differentiated and separated from each other. This new capability will help to permit the selection of single, nonheterogeneous populations of a virus which can then be used for experimental vaccine development. This chromatographic technique is being applied to another alphavirus, CHIK; it may work as well for RVF and perhaps even the arenaviruses.

Initial studies were begun to develop bacterial vaccines against melioidosis and Legionnaires' disease. The latter has shown some initial promise in that the whole killed organism, or soluble extracts, protect against challenge with the live organisms.

Another important facet of the vaccine development program includes the need for highly standardized, carefully monitored laboratory animal data when experimental vaccines are initially tested; programs have been developed to do this. The requirement for extensive record-keeping and quality control animal measurement data is essential so that acceptable and complete data can eventually be submitted for approval to support the value of a new vaccine prior to its initial testing in man.

Vaccine Adjuvant Studies

A portion of vaccine development research is devoted to the study of adjuvants that could improve the immunogenicity of marginally effective vaccines. Adjuvants selected for applied study emphasized those with a potential for eventually being approved for use in man. These included 2 interferon-inducers, lysine-stabilized poly(I)·poly(C) [poly-(ICLC)] and an analog of the drug, tilorone hydrochloride. Other adjuvants included dialyzable leukocyte extracts, biodegradable oil-water emulsions, and muramyl dipeptide. In the studies using tilorone analogs or poly(ICLC), an adjuvant-vaccine combination significantly enhanced resistance in mice against all test virus infections when compared to control mice receiving vaccines without adjuvant. Similarly, a peanut-oil, lipid emulsion given with VEE vaccine was effective in both mice and monkeys on the basis of both enhanced resistance to virus challenge and development of higher serum neutralizing antibody titers.

In more basic studies, the various experimental adjuvants have been compared to the effects achieved with complete Freund's adjuvant which, although it is not safe for use in man, has long acceptance as being the most potent product available for enhancing the immunogenicity of weak antigens in animals. In these comparisons, the Freund's adjuvant was found to potentiate both cellular and humoral primary immune responses,

whereas muramyl dipeptide in oil appeared to improve the primary cell-mediated immune (CMI) responses, but potentiated only anamnestic humoral responses to inactivated VEE vaccine antigens.

Immunologic Responsiveness Studies

Another sizable portion of the in-house program involved studies to determine how immune responses could be measured and manipulated so that efficacious safe vaccines could be developed. These studies included investigations into the relative efficacy of experimental vaccines administered via different routes, the responsiveness of cell-mediated immune mechanisms to vaccines or infection, the effects of "selective" and general immunosuppression (such as that produced by acute irradiaition), the immunologic functions of macrophages and lymphocytes and the production of immune complexes in plasma.

Because BW defensive measures must consider the exposure of troops via an aerosol, research was conducted to determine optimal immunologic methods for generating protective immunity on mucosal surfaces throughout the respiratory tract. These included studies of aerosol immunization to tularemia via the lungs and studies of airborne infections with JE in monkeys and mice, and Pseudomonas pseudomallei (melioidosis) infections in mice and hamsters. Mice surviving an initial infection with JE were solidly protected against rechallenge, but neither killed virus vaccine nor the passive administration of immune serum protected them. Aerosol infections also produced lethal melioidosis in hamsters and squirrel monkeys. Initial studies suggested that some protection against respiratory melioidosis could be provided by killed cell vaccines.

To evaluate CMI, comparisons between leukocyte-adherence inhibition and macrophage-migration factor tests were followed during tularemia infection in mice. Other studies in mice identified, through Mishell-Dutton assays, the extent of participation of B- and T-lymphocytes and macrophages in response to vaccination with either live or killed tularemia vaccines. Methods were also devised to quantitate delayed hypersensitivity reactions and to detect the magnitude of "suppressor" or "helper" functions of different transfused lymphocyte populations in mice inoculated with the live, attenuated tularemia vaccine. Protection against highly virulent tularemia organisms appeared to require both T- and B-lymphocyte activity. In other studies, the participation of CMI mechanisms were studied in nude mice because of their congenital lack of thymic functions. This approach was especially valuable in attempting to determine why some of the arenavirus infections were capable of producing delayed lethal encephalitis. Studies using nude mice led to the conclusion that lethal encephalitis caused by Tacaribe virus was immune-mediated and dependent upon the presence of intact functioning T-lymphocyte mechanisms.

Methods were developed to study the production of immune complexes in the circulation following the exposure of immunized or nonimmunized animals to virulent microorganisms. This new approach uses isotachophoresis to identify the complexes through their patterns of migration in an electrical field.

Additional basic studies were conducted to define the role of lymphocytes and macrophages in immune mechanisms. A series of novel studies defined, for the first time, the physical and chemical requirements for lymphocyte chemotaxis under in vitro conditions. Lymphocytes migrating between agarose and glass layers were attracted by gradients of chemotactic factors which differed chemically from those known to attract polymorphonuclear leukocytes.

Because some anti-inflammatory agents may affect the induction of immunity through their cytoskeletal effects, the cytoskeletal probes (cytochalasin-A and colchicine) were used to define the role of cellular microfilaments and microtubules in regulating lymphocyte movement through lymph nodes, during cell cooperation actions, and during their recirculation throughout the body. It was also shown that the high endothelial cells of lymph nodes venules could remove antigen-receptor complexes from the surfaces of recirculating lymphocytes without damage to the lymphocytes. The "scrubbing" process was postulated to be of value in exposing new receptors which rendered the lymphocyte more responsive to subsequent antigenic stimulation.

Studies of macrophage function continued to use cells collected from the pulmonary alveoli of cynomolgus monkeys during pulmonary infection. The IV administration of the drug, glucan, prior to an aerosol exposure to an infectious organisms was found to exert a nonspecific stimulating effect on the monocyte-phagocyte system; this treatment materially reduced mortality during intracellular infections, such as tularemia in rats. Mechanisms were developed for studying the detailed function of macrophage organelles following bacterial phagocytosis. The mannose-rich capsule of tularemia organisms was found to be important in allowing the organisms to be destroyed within macrophages. However, these phagocytic cells were capable of taking up virulent tularemia organisms whether or not the capsule had been removed.

Still other immune responses were studied in immunosuppressed animals. Suppression studies associated with high doses of whole-body irradiation were limited during FY 78 because the USAMRIID high voltage x-ray source could no longer be repaired. In other suppression studies, cyclophosphamide treatments given prior to immunization with various types of vaccines were used in an effort to abolish humoral antibody responses, while at the same time allowing CMI functions to be preserved, or even potentiated if these were being repressed by blocking antibodies. When treated with cyclophosphamide, animals showed a loss of immune protection when challenged with VEE virus, unchanged protection against tularemia, but enhanced protection when challenged with rickettsial organisms, including those which produced Q fever and tick-borne spotted fever.

Pathogenesis Studies

Pathogenesis studies in animal infections are needed to test new vaccines, diagnostic techniques and therapeutic measures. During the year, USAMRIID attempted to create suitable model infections in laboratory animals for Legionnaires' disease, KHF, LAS, Congo/Crimean hemorrhagic fever, RVF, and infections with arenaviruses less dangerous than LAS, i.e.,

Pichinde and Tacaribe. In searching for a representative model, especially for such a difficult disease to study as KHF, a large variety of both common and rare laboratory animal species were tested, including some, such as cotton rats, vesper mice and voles, which are not available commercially and must be bred in-house. Such studies also call for different subhuman primate species as well as inbred strains of the more common laboratory rodents.

Emphasis has also been continued on defining the unique pathogenic patterns of illness produced by aerosolized organisms, including bacterial bronchopneumonias, lobar pneumonias, and viral (e.g., JE), which appear capable of entering the central nervous system from the nasal mucosa through olfactory nerves traversing the cribriform plate.

Additional studies have been conducted to define some of the physiologic and biochemical responses that accompany infectious diseases. These included studies of body fluid and electrolyte shifts in yellow fever and the hepatorenal failures which may accompany various infections. This work has been strengthened by the development of computerized techniques for collecting and recording data from many on-going simultaneous physiologic measurements. Biochemical studies included additional work to define the mechanisms used to provide metabolizable energy for the infected host and to characterize the role of the liver in producing the large variety of new "acute-phase reactant" serum glycoproteins and hepatic metallothioneins during a variety of different infections.

Diagnostic Studies

Diagnostic studies covered several different areas. A major new research thrust was initiated in order to establish and maintain immunologically based diagnostic capabilities for a wide variety of virus diseases of special importance to the USAMRIID medical defense program. The new approaches involved the development of improved fluorescent antibody technology, radioimmunoassay (RIA) methods, enzyme-linked immunosorbent assay (ELISA), and chemiluminescent-immunoassay techniques, all of which are being compared with each other, as well as with the more time-consuming microbiological assay methods, such as viral plaque neutralization tests, which can be employed to provide validation for each new method. Spot tests on microscopic slides for fluorescent antibody identification of a large number of viruses were prepared, standardized and tested for safety. Additional slides to test for new viruses are currently being developed both in-house and under contract.

Unique new aspects of diagnostic work included a confirmation of the reliability of the fluorescent antibody techniques for the diagnosis of KHF. Serum specimens obtained from nephrosonephritis patients in 3 Scandinavian countries were positive for the KHF antigen, providing important new evidence that the diseases are linked or are identical. Tissue culture growth of KHF virus was achieved for the first time. There were also initial breakthroughs showing that the new RIA and ELISA techniques had great usefulness for virological diagnosis. ELISA techniques have also been developed for bacterial organisms and toxins and appear to provide a more rapid method for diagnosis of Legionnaires' disease.

A major accomplishment was the development of a fully contained P4-level diagnostic laboratory, previously described under Clinical Studies. This included the training of personnel necessary to permit all diagnostic microbiological and clinical laboratory samples to be handled and processed in fully contained biological safety laboratories, with investigators and technicians working in total-body positive-pressure safety suits.

Additional approaches in the diagnostic area included further studies to determine if changes in chemiluminescence or the biochemical composition of white blood cells or blood platelets could be used as early indicators of the presence of an infectious disease. Further progress was made on computerizing the various diagnostic approaches of a biochemical nature to determine if diagnostic patterns might emerge which would be of greater value than changes in single biochemical parameters alone. Finally, a new technique using isotachophoresis was developed to permit the isolation of immune antigen-antibody complexes from serum that could possibly prove to be of value in the diagnostic area.

Therapy Studies

Therapy studies involved important new work on antiviral compounds against unique, highly dangerous viruses. Work was continued on the use of aerosolized antibiotics in pulmonary infections, and the use metabolic and physiologic approaches for providing supportive therapy. This latter need is especially important during overwhelming infections, including those complicated by disseminated intravascular coagulation or the development of hepatorenal failure.

A dramatic achievement was the discovery that ribavirin was effective for therapy of such dangerous infections as those caused by members of the arenavirus group of hemorrhagic diseases, RVF, and possibly yellow fever. Earlier work with the interferon-inducing drug, poly(ICLC), was continued and new studies were introduced to determine if antioxidant drugs had value in infections due to lipid-containing viruses, but the major emphasis was placed on studying the therapeutic effectiveness of ribavirin. While ribavirin was previously shown to have prophylactic efficacy, the major new finding showed that it was effective even though treatment was not begun until after the onset of clinical illness due to Machupo virus in monkeys and guinea pigs, RVF infection in mice and hamsters, and to a limited degree, yellow fever infection in monkeys. Work is on-going to determine the localization of ribavirin within tissues and the molecular mechanism of its antiviral activity within cells. In addition, arrangements were made to obtain new analogs of ribavirin that could cross the blood-brain barrier.

Studies to extend knowledge in the areas of aerosol therapy with antibiotics indicate that pulmonary concentrations of drugs, such as kanamycin, can reach a therapeutic level without dangerous accumulation in the kidneys. Aerosol therapy was more effective against bronchopneumonia in animal models than against frank lobar consolidations.

Since antimicrobial agents are not available for all lethal infections, continued emphasis was placed on improving metabolic and physio-

logic support and correction of imbalances. It proved possible to control many of the infection-induced abnormalities in amino acid, protein, carbohydrate, insulin, free fatty acid, and ketone metabolism during experimental infections by the therapeutic administration of appropriate metabolic substrates. Studies are underway to control hepatorenal failure by using hemoperfusion techniques and correction of salt and water imbalances.

The catastrophic complication of disseminated intravascular coagulation during a variety of bacterial, viral and parasitic infections was approached with a protease inhibitor, aprotinin, as a therapeutic drug to influence the kinin system. Studies in monkeys were conducted to determine the pharmacokinetics of aprotinin during experimentally induced infection and to determine the effects of aprotinin on blood clotting mecha nisms.

Bacterial Toxin Studies

Studies included the botulinum neurotoxins, anthrax toxins, several staphylococcal enterotoxins, enterotoxins produced by cholera and Shigella spp., diphtheria exotoxin, and Pseudomonas exotoxin A and exoenzyme S.

In a major new program, a USAMRIID effort was initiated to produce a multivalent botulinum toxoid, since the capability to produce additional quantities of the older vaccine no longer exists elsewhere in the free world. This program made excellent progress. The representative, previously used strains of toxin-producing organisms were obtained. Initial efforts began with botulinum neurotoxin A and were highly successful, allowing for the production of sufficient volumes of culture to permit extraction and purification by modern chemical techniques of milligram quantities of neurotoxin A. Modern techniques were also used to toxoid this into a safe and immunogenic vaccine.

In addition, a program was initiated to collect many liters of high-titer human antibotulinum plasma from individuals who previously had been immunized repeatedly with the existing polyvalent botulinum toxoid. Approval was obtained from the Army and the Food and Drug Administration to use this human hyperimmune botulinum plasma for the therapy of acute botulism if this should become necessary. In addition, arrangements were made under contract to convert large quantities of the plasma into hyperimmune botulinum immunoglobulin.

Studies on anthrax toxins and protective immunogens had been at a virtual standstill for at least a decade. During FY 78, USAMRIID entered this field in an attempt to produce a more effective immunogen that could be used in man for production of protective immunity. The currently available vaccine is a crude culture filtrate which requires 18 mon for the primary vaccination series. Anthrax organisms produce at least 3 poorly characterized exoproteins: protective antigen, edema factor and lethal factor. It is necessary to reestablish methodology to culture selected strains, in order to produce sufficient exoproteins to allow for their eventual differentiation, purification and toxoiding by modern techniques.

Much basic work was accomplished in defining the tertiary and secondary structures of staphylococcal enterotoxin B (SEB) and its component peptides, and in comparing them with comparable portions of types A (SEA) and $C_1$ ($SEC_1$) enterotoxins to define which portions of the protein molecules were immunogenic and which caused toxicity. In addition, a capability for sequencing proteins was established. Initial steps were taken to ascertain the correct amino acid sequences of staphylococcal toxins with emphasis on $SEC_1$ and the dermatolysins. In other basic work, the mechanism used by staphylococci to excrete their exoprotein toxins was shown to depend upon the fatty acid composition of external membranes. Evidence was also obtained that a proteinase was required to release the toxin into the culture medium.

The mechanism of action of SEB was studied with respect to the exchange and loss of fluid across the intestinal mucosa of the rabbit. A potential breakthrough in therapy emerged, when it was shown that SEB could be taken up and bound by activated charcoal. Initial attempts were then made to utilize this concept in hemoperfusion studies, which would allow toxin in the blood stream to be removed during extracorporeal circulation through a charcoal filter. Exploratory physiologic studies were also performed to determine if either cholera or Shigella enterotoxins were toxic following exposure by the parenteral or aerosol route, rather than the usual gastrointestinal route.

Progress was made in defining the nature of Pseudomonas exotoxins (PE). Additional studies for scaled-up production and purification of PE-A was made, with toxoiding mechanisms being developed. The resultant toxoid provided partial protection against homologous infection in burned mice, whereas, little protective efficacy was demonstrated in burned, infected rats. Attempts to purify another Pseudomonas exoprotein, exoenzyme S, had initial successes.

Major advances were made in studying the specific toxic mechanism of action of PE and the closely related diphtheria exotoxin (DE). In vitro studies in cultured cells employed radioactively labeled exotoxin molecules to demonstrate for the first time that specific cellular receptors exist for each of these exotoxins. The presence of a specific cellular receptor is necessary to allow the toxin to enter a susceptible cell. The USAMRIID demonstration that such receptors exist on susceptible cells has already been followed by identification of drugs which prevent the binding of toxins to cells, thus preventing toxin entry and lethal action. In closely related, equally novel work, both DE and PE have been conjugated with either ferritin or colloidal gold in order to visualize the toxin receptors on cells by electron microscopy.

## Rickettsiology Research

Rickettsiology studies emphasized continuing attempts to produce, improve or evaluate rickettsial vaccines. Other research involved pathogenesis studies by light and electron microscopy to elucidate the nature of the earliest stages of the vascular endothelial lesions which characterize many rickettsial diseases.

Q fever studies proceeded along 3 paths. Additional data were gathered in monkeys to evaluate the relative efficacy of the inactivated phase I Q fever vaccine prior to initial testing in man. These tests, used a newly standardized cynomolgus monkey model in which Q fever infection closely resembles the illness seen in man, with comparable interstitial pneumonia, hematologic, physiologic and immunologic responses. Additional attempts were made to isolate purified components of Coxiella burnetii in hopes of identifying specific components that were highly immunogenic and effective in preventing Q fever without having the undesirable side-effects of the existing phase II Q fever vaccine. Soluble phase I antigens of C. burnetii were treated with various enzymes, including proteinase, lipase and lysozyme, with the last producing an immunogen which appeared to have reduced reactogenicity.

A third line of study involved the infection of athymic, nude mice and normal euthymic control mice with small-particle aerosols of C. burnetii. Euthymic mice recovered rapidly from the infection and cleared rickettsiae from peripheral blood and spleen within 14 days, whereas, the immunodeficient athymic mice showed continued presence of rickettsiae in blood and spleen for at least 60 days. This finding adds to the evidence concerning the importance of cell-mediated immunity in host resistance against Q fever.

Additional studies of the new RMSF vaccine were conducted in volunteers, as discussed in Clinical Studies. In addition, studies in laboratory animals were continued to produce a broadly protective vaccine against all varieties of spotted fever rickettsiae. Studies in guinea pigs showed that the new RMSF vaccine prepared for human use was protective against virulent strains of Rickettsia rickettsii obtained from diverse geographical sources. In addition, cross-protection studies were conducted in guinea pigs and mice relating to the immunologic similarities of Rickettsia spp: rickettsii, conorii, sibirica and akari. Although none of these species produces lethal illness in guinea pigs, they each induce cross-protection against infection by other members of the spotted fever group, as shown by prevention of a febrile response. A lethal R. akari infection was found to occur if BALB/c mice were used as an animal model, such mice being protected from death by the vaccine.

In a final aspect of the studies on immunization against spotted fever rickettsiae, 2 additional approaches were used. Soluble immunogenic antigens derived from suspensions of whole rickettsiae were found to protect laboratory animals. Limited success was achieved in attempting to use a relatively avirulent organism, Rickettsia montana as a live rickettsial vaccine.

Since spotted fever rickettsiae produce vascular lesions, studies were conducted using light and electron microscopy to demonstrate the earliest changes in endothelial cells in infected tissue culture cells or in the vascular endothelial cells of arteries obtained from guinea pigs. As an alternative approach, a chick embryo model was used to study the initial lesions in vessels of the allantoic membranes. In each instance, the rickettsiae were always present in the earliest lesions. In the egg, vascular lesions appeared before the embryo was immunologically

competent and before proteins of the complement and coagulation systems had developed. These data, in combination, suggest that the vascular lesions can best be explained by direct action of the rickettsial organisms *per se*.

In one final area, the methods used successfully to produce a whole-organism spotted fever vaccine from tissue culture were extended to *Rickettsia prowazekii* to determine if an improved epidemic typhus vaccine could be prepared. This new tissue culture vaccine was at least as efficacious as the presently available commercial vaccine. Further tests are planned for comparing these products in subhuman primates.

To summarize, this brief review of USAMRIID progress during FY 1978 illustrates the success of a multidisciplinary approach in fulfillment of the mission of the Institute. It becomes obvious that a medical research program directed toward BW defense involves every aspect of microbiology, aerobiology and immunology.

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